UNIVERSIDADE FEDERAL DO PARANÁ

AMANDA BOMBASSARO

VIRULENCE ASPECTS OF *FONSECAEA* SPECIES ASSOCIATED TO DISSEMINATED INFECTION BASED ON THE COMPARATIVE GENOMIC ANALYSIS AND IN VIVO ASSAYS

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RESUMO

Fungos melanizados são caracterizados por pigmentação escura na parede celular. Estima-se que mais de cem espécies de fungos pretos possam causar doenças, que podem ocorrer por via de inoculação traumática desses agentes nos tecidos. Este grupo de fungos são membros da ordem Chaetothyriales e são relatados como agentes de cromoblastomicose e feo-hifomicose. A cromoblastomicose é descrita como uma infecção subcutânea que apresenta lenta evolução de lesões verrucosas nos tecidos, que formam células septadas nos tecidos, denominadas células muriformes. Enquanto, a feo-hifomicose é definida pela presença de hifas nos tecidos de vários órgãos, como fígado, coração, rim, cérebro e outros. Mas o neurotropismo ocorre com frequência notável por esse grupo de fungos, possivelmente devido à capacidade de algumas espécies de metabolizar hidrocarbonetos aromáticos. As leveduras negras *Fonsecaea monophora* e *Fonsecaea pugnacius* compartilharam o mesmo perfil de patogenicidade, sendo relatadas como agente de cromoblastomicose e agente causador de infecção cerebral primária em humanos. Este trabalho teve como objetivo gerar dados de seqüenciamento de genoma e analisar comparativamente os genomas das espécies de *F. monophora* e *F. pugnacius* para entender os mecanismos de biologia e virulência desses agentes. Foi realizada uma análise genômica comparativa das linhagens de irmãos: *F. monophora* e *F. pugnacius*, incluindo a montagem do genoma de *F. pugnacius.* O tamanho dos genomas são 34,21 e 34,8 Mb respectivamente, e o *core* do genoma dessas espécies compreende quase 70% dos genes. A similaridade do repertório de enzimas associada à ocorrência de fatores de virulência sugeriu uma tolerância geral a condições extremas, o que pode explicar a tendência oportunista dessa espécie como as demais espécies do gênero *Fonsecaea*. A virulência foi testada nos modelos: *Tenebrio molitor* e Balb/C para apoiar essa hipótese. A capacidade de sobrevivência de ambos os fungos no interior do *Tenebrio molitor* foi confirmada por análise histopatológica e pela presença de melanina no tecido hospedeiro. Embora *F. pugnacius* tenha sido isolado do cérebro de BALB/C após infecção intraperitoneal, os níveis de citocinas não foram estatisticamente significativos, corroborando com a hipótese de ser agente oportunista. Uma dupla capacidade ecológica pode ser concluída a partir da presença de vias metabólicas para eliminação de nutrientes e extremotolerância, combinadas com a capacidade de infectar hospedeiros humanos.

Palavras-chaves: Virulência. Genomas. Fungos. Cromoblastomicose.

ABSTRACT

Black yeasts-like are fungi characterized by dark pigmentation in the cell wall. It has been estimated that more than a hundred species of black fungi can cause disease, which can occur in the injured tissues by fungi. This group of fungi are members of Chaetothyriales order and are related like as agents of chromoblastomycosis and phaeohyphomycosis. Chromoblastomycosis is characterized as a subcutaneous infection present slow evolution of verrucous lesions in the tissues, which form septate cells in the tissues, called muriforms cells. While, phaeohyphomycosis is defined by the presence of hyphae in the tissues of several organs such as liver, heart, kidney, brain and others. But the neurotropism occurs with remarkable frequency in the fungal group, possibly due to the ability of some species to metabolize aromatic hydrocarbons. The black yeasts *Fonsecaea monophora* and *Fonsecaea pugnacius* shared the same pathogenicity profile, being reported as a chromoblastomycosis agent and causal agent of primary brain infection in humans. This work aimed generate genome sequencing data and comparative analyze the genomes of *F. monophora* and *F. pugnacius* species to understand the biology and virulence mechanisms these agents. A comparative genomic analysis of sibling strains: *F. monophora* and *F. pugnacius* was undertaken, including the genome assembly of *F. pugnacius*. The genome size of strains are 34,21 and 34,8 Mb and the core genomes of those species comprises almost 70% of the genes. The similarity of enzymes repertory associated with the occurrence of virulence factors suggested a general tolerance to extreme conditions, which might explain the opportunistic tendency of this species like the others *Fonsecaea* sibling species. Virulence was tested in the *Tenebrio molitor* model and Balb/C were performed in order to support this hypothesis. The capacity of both fungi to survive inside *Tenebrio molitor* was confirmed by histopathological analysis and by presence of melanin in host tissue. Although *F. pugnacius* was isolated from brain in a murine model following intraperitoneal infection, cytokine levels were not statistically significant, indicating a profile of an opportunistic agent. A dual ecological ability can be concluded from presence of metabolic pathways for nutrient scavenging and extremotolerance, combined with a capacity to infect human hosts.

Keywords: Virulence. Genomes. Fungi. Chromoblastomycosis.

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CHAPTER I

Literature Review

1. GENERAL INTRODUCTION

Melanized fungi belonging to the family Herpotrichiellaceae are extremely important microorganisms from an ecological and clinical point of view. These common saprobic organisms have high adaptability and a compound life cycle and pathogenicity potential, thus justifying the low environmental occurrence and frequency of infection in animal hosts. This potential for infection appears to be polyphyletic and consequently differs between species. Recurrent and consistent infections indicate a possibility of host adaptation and are known to be caused by a few species that are often very morphologically similar. Thus, is fundamental to understand the link between disease and the environmental ecology of the agent (GOMES et al., 2016).

Among the infections caused by these organisms, chromoblastomycosis is characterized by the appearance of verrucous-looking skin and subcutaneous lesions that may emerge as tumor-like ulcerative eruptions (BRITO & BITTENCOURT, 2018). The traumatic inoculation of spines or wood chips is the source of infection (FORNARI et al., 2018). Chromoblastomycosis is mainly a tropical or subtropical disease that may affect individuals with certain risk factors around the world (QUEIROZ-TELLES et al., 2017). In Brazil, the chromoblastomycosis cases have been reported in studies from 1914, with high levels in 1999, and new cases are being related every year (DONG et al., 2014; GOMES et al., 2016; QUEIROZ-TELLES et al., 2017).

Several genus present causal species of chromoblastomycosis such as *Rhinocladiella, Phialophora, Cladophialophora, Exophiala* and *Fonsecaea* (QUEIROZ-TELLES et al., 2015). The molecular epidemiology of the disease has revealed that the genus *Fonsecaea* presents four causal species, distributed throughout the endemic areas of the world: *Fonsecaea pedrosoi, F. nubica, F. monophora* and *F. pugnacius* (NAJAFZADEH et al., 2011a; AZEVEDO et al., 2015). While *F. pedrosoi* and *F. nubica* are strictly associated with chromoblastomycosis and formation of muriform cells, *F. monophora* and *F. pugnacius* have also been reported in brain and other organ infections (VICENTE et al., 2017).

Regarding the *Fonsecaea monophora* is associated to chromoblastomycosis with formation of muriform cells, being the predominant causal agent in China (FRANSISCA et al., 2017). This species may also present neurotrophism in the human host being an agent of primary brain infection, in which neurological symptoms are the first indications of infection, once the dissemination occurs

unnoticed (STOKES et al., 2017).

However, *F. pugnacius* is the unique species described causing lesions with muriform cells and brain abscesses in the same immunocompetent patient. Histopathological analysis of this case reported the presence of muriform cells in a skin sample and hyphae in a brain sample, indicating a change in cell conformation to an invasive morphology (AZEVEDO et al., 2015).

Questions concerning the route of infection, causal agents and the relationship of the agents with the variability of clinical cases and consequently variations, related to the response to treatment and cure of the disease, as well as the virulence potential of the isolates from human origin are not totally clarified. These disease represents a public health problem for the population of certain Brazilian regions and tropical areas. Due to this scenario, the understanding of the genes involved in the mechanisms of virulence, pathogenicity and a better understanding of the biology of these agents, making possible the development of biomarkers for diagnosis, epidemiological studies and targets for drug development and treatment protocols are an emergency demand.

Therefore, the present work aimed to generate and analyze genomic data and to characterize the karyotype of the species that cause disseminated Chromoblastomycosis: *F. monophora* and *F. pugnacius*, aiming to obtain data to understand the genes involved in the mechanisms of virulence, pathogenicity and greater understanding of biology and these agents.

2. OBJECTIVES

2.1 GENERAL

Generate genome sequencing data and to perform genomic comparative analysis of *Fonsecaea monophora* and *Fonsecaea pugnacius* species to understand the biology and virulence mechanisms these agents.

2.2 SPECIFICS

• To perform DNA extraction, genomic sequencing, assembly and annotation of the partial genome of *F. pugnacius.*

• To explore the data generated by genomic sequencing, assembly and annotation of the draft genome of *F. monophora* (data generated in the master's degree) and *F. pugnacius.*

• To elucidate the main metabolic pathways of the study species.

• To identify genes and mechanisms of pathogenicity and virulence.

• To study the homology of nucleotide and amino acid sequences among species of the genus *Fonsecaea*.

• To characterize the karyotype of the species *F. monophora* and *F. pugnacius*.

3. LITERATURE REVIEW

3.1 CLINICAL IMPORTANCE OF BLACK YEASTS

Black yeasts like are fungi whose main characteristic the presence of melanin in the cell wall of vegetative and reproductive cells. This group shows great adaptability, allowing them to tolerate a wide variety of hostile conditions such as high temperatures, UV radiation, high concentrations of salt, desiccation, variations in pH and nutrient deficiency (SEYEDMOUSAVI et al., 2014).

These fungi form a heterogeneous group regarding the taxonomy and phylogeny that covers Ascomycetes; e.g., members belonging to the order Chaetothyriales (SELBMANN et al., 2013) in the family Herpotrichiellaceae, which contains ecologically and clinically relevant microorganisms, which have pathogenicity potential in animal hosts. The yeasts of this family present slow growth, initially yeast-like, and over time, the texture of the colony becomes velvety with pigmented hyphae. The main species belong to the genus *Exophiala, Cladophialophora, Fonsecaea* and *Phialophora,* (QUEIROZ-TELLES et al., 2017).

According to Seyedmousavi et al. (2014), most melanized fungi are common soil saprobes and decomposing plants. However, several of these agents are considered opportunistic colonizing human and animal hosts (QUEIROZ-TELLES et al., 2017). In this context, it has been previously emphasized that the infection basically depends on three factors to establish: host resistance, inoculum quantity and fungal virulence (ALVIANO et al., 2004).

An important factor of virulence in black yeast like is thermo-tolerance (HOOG et al., 2011). Increased reports of fungal diseases caused by these agents in domestic and wild animals, invertebrates and warm-blooded or cold-blooded vertebrates (SEYEDMOUSAVI et al., 2014) demonstrate that temperature may be a determining factor in host choice (HOOG et al., 2011; VICENTE et al., 2017). Recently, the route of infection by insect was proven by JACOMEL et al. (2020) demonstrating that social insects, specifically ants, bees and termites, provide an adequate habitat for these fungi and be an infectious source for mammals.

Melanin also appears to contribute to the virulence of these organisms by enhancing the resistance of fungal cells in response to the immune system of the host. This pigment probably makes possible three mechanisms: the reduction of phagocytosis, the protection of fungi against the hydrolytic enzymes produced by

macrophages and against the derivatives of oxygen and nitrogen. Although the mechanisms of invasion of the immune system and permanence in human tissues are unknown, it is known that the production of melanin protects the fungus against the activation of macrophages and neutrophils (VICENTE et al., 2017).

The polysaccharide synthesis represents a virulence factor in black yeasts like, an alternative strategy under unfavorable conditions (BREITENBACH et al., 2018). Alviano et al. (2004) demonstrated that *F. pedrosoi* cell wall polysaccharides were able to induce a granulomatous reaction in animals and are related to the pathogenicity of the species. A polysaccharide found in the cell wall of some species of the genus *Fonsecaea* is α-1-3-glucan, which are presented in all the dimorphic pathogenic fungi studied and related to the virulence of some, such as *Histoplasma capsulatum* and *Paracoccidiodes brasiliensis* (TANG et al., 2018).

It has been estimated that more than a hundred species of melanized fungi can cause disease, which can occur in the injured tissues like hyphae, pseudohyphae, yeast cells or any combination of these forms (QUEIROZ-TELLES et al., 2015). The pathogenic and opportunistic melanized fungi of humans and animals emerge as agents of eumycetomas, chromoblastomycosis and phaeohyphomycosis; which can remain in the traumatic inoculation sites, internalize or disseminate (QUEIROZ-TELLES et al., 2015).

Mycetoma consists of the nodules formation containing grains in tissues. It is characterized as a chronic and long-term infection. When caused by fungi it is called eumycetoma and is related to several etiological agents. Infection occurs from traumatic inoculation and there is a significant increase in limb volume, with deformity and functional limitation. The disease is classified as subcutaneous, localized with rare spread (QUEIROZ-TELLES et al., 2015). Eumycetomas have a fibrous texture, causing the response to conventional treatments to be impaired, since the antifungal drugs do not reach the necessary concentrations at the site of infection. Therefore, the current treatment consists of the use of terbinafine, voriconazole and posaconazole; allied to surgical treatment (SAMPAIO & RIVITTI, 2014).

Chromoblastomycosis is characterized as a subcutaneous infection, caused by several black fungi, which form septate cells in the tissues, called muriforms cells. The disease presents the slow evolution of verrucous lesions in the tissues (QUEIROZ-TELLES et al., 2017).

Phaeohyphomycosis includes opportunistic mycoses caused by black yeast like, which occur from the superficial, cutaneous, subcutaneous and even disseminated forms, without the formation of muriform cells. In this infection the

agents appear in the tissues as yeast cells or vesicular elements, pseudohyphae, septate and toroid hyphae (THOMAS et al., 2018). In 2006, Esterre and Queiroz-Telles, related the occurrence mainly in regions of tropical climate such as Madagascar, northern Venezuela, and Amazon region of Brazil, but there are also reports of the occurrence of cases in temperate regions. It is a sporadic, cosmopolitan infection that affects immunocompetent and immunosuppressed individuals; which can affect animals of various species (REVANKAR et al., 2017). In immunocompromised individuals, the distinction between chromoblastomycosis and phaeohyfomycosis may become difficult due to the deficiency of the immune response of these hosts, which probably does not lead to adequate conditions for the formation of muriform cells (QUEIROZ-TELLES et al., 2017).

Cutaneous and subcutaneous phaeohyphomycosis occurs mainly in immunocompromised adults, often in patients between 50 and 80 years of age (ARCOBELLO & REVANKAR, 2020). The most frequent etiological agents are of the genus *Exophiala*, *Alternaria* and *Wangiella*, being the most reported species *E. jeanselmei* (ROSSETTO et al., 2010). Cerebral phaeohyphomycosis has a wide geographic distribution, reported from Europe, Asia, Africa, North America and South. The main agents have been *Cladophialophora bantiana, Exophiala dermatitidis* and *Rinocladiella mackenziei* (MORENO et al., 2018a). In animals, the disease has occurred in cats and crab (NAJAFZADEH et al., 2011b; VICENTE et al., 2012).

Disseminated phaeohyphomycosis is a rare infection; however, its incidence has been increasing and often fatal (KANTARCIOGLU et al., 2017). Between the 1960s and 1980s, 11 cases were reported, 8 of which involved immunocompetent patients; as early as the 1990s, 45 cases were reported, of which 41 were patients with some type of immunodeficiency, usually induced by chemotherapy QUEIROZ-TELLES et al., 2015). Recently, many cases have been attributed to *Rhinocladiella mackenziei* (ALABDELY et al., 2020; MORENO et al., 2018a) and others have been linked to mutations in the *CARD9* gene (HUANG et al., 2019; WANG et al., 2019).

This suggests that the main reason for the increase in the number of cases of the disseminated form of the disease may be immunodeficiency caused by intense drug treatments (QUEIROZ-TELLES et al., 2015). Therapy is not standardized and is based primarily on clinical experience from descriptive case reports. The development of new antifungal agents and the combination of therapies may be an option for a better treatment of this infection (ARCOBELLO & REVANKAR, 2020).

3.2 CHROMOBLASTOMYCOSIS: CLINICAL ASPECTS, DIAGNOSTIC METHODS, EPIDEMIOLOGY AND ETIOLOGY

Chromoblastomycosis was described in 1914 by Max Rudolph, where reported the first six cases of the disease (RUDOLPH, 1914). Recently, considered a neglected disease by the World Health Organization (WHO, 2016). The chromoblastomycosis is classified as a mycosis of implantation, caused by the insertion of the agent into the tissue through physical trauma. In addition, is a chronic infection caused by some black yeasts like starts from a primary lesion, beginning at the site of inoculation of the etiological agent, with chronic skin involvement may advance to the subcutaneous tissues with a granulomatous, purulent and fibrotic tissue reaction. The main risk factors associated with chromoblastomycosis are adult, male sex, rural work or outdoor activities, lack of protective footwear, gloves or clothing, and poor nutrition and hygiene habits (QUEIROZ-TELLES et al., 2017). When the immune response of the host is initiated, the formation of brownish round differentiated cells with thick walls occurs, appearing in isolation or in a clustered fashion. These cells present binary division and are denominated muriform cells, sclerotic or corpuscles (BRITO A. C. & BITTENCOURT, 2018).

The lesions are clinically polymorphic and may become recalcitrant to therapy, extremely difficult to eradicate, if not recognized in the early stages (SARKAR et al., 2017). Classically defined by type and severity, the lesions can be: nodular, tumoral (cauliflower-like), verrucous, scarring, and plaque. The different levels are related mainly to the time of infection, the site, the patient's hygienic, the antifungal therapy, and the immune response, including primary immunodeficiencies such as genetic mutations (QUEIRROZ-TELLES, 2017). The lesions are dry and do not cause pain, due to this it is very common cases of patients who present lesions for many years (QUEIROZ-TELLES et al., 2017). The internal organs may be invaded by some etiological agents of this disease and sometimes the infection affects the brain, and the lesions may be isolated or multiple; and appear as encapsulated abscesses or generalized inflammatory infiltrations (SARKAR et al., 2017).

Due to the diverse morphology of the lesions, the diagnosis is made by the observation of muriform cells in direct and/or histopathological mycological examination, as well as by the isolation and identification in the culture (BRITO A. C. & BITTENCOURT, 2018). Muriform cells can be easily observed in mycological exams directly from scrapes of lesions prepared with potassium hydroxide (KOH) (10-40%), since the sensitivity of this test reaches 90 and 100%. In histological analyzes, the lesions are characterized by epidermal hyperplasia alternating with

areas of atrophy and inflammatory cell clusters forming epidermal abscess. Granulomatous reaction with different degrees of fibrosis can be found at the dermal level (DINIZ et al., 2019).

In spite of Zeng and Hoog (2008) described the Internal Transcribed Spacer of the DNAr, Tubulin and Actin gene sequences of 156 *E. spinifera* strains and concluded that data from internal transcribed spacer sequences are reliable for phylogenetic reconstruction and identification of the species of this clade. In culture medium, the morphological similarity between black yeasts makes it difficult to determine the taxonomic classification. Thus, the identification of the species is based on molecular methods and specific genes. For Herpotrichiellaceous, the molecular analyzes indicated are the sequencing of the small subunit, internal transcribed spacer, elongation factor-1, tubulin and actin (GOMES et al., 2016).

Chromoblastomycosis is a cosmopolitan disease but the most cases occur in regions with the highest prevalence in tropical and subtropical regions, such as Madagascar, Africa, Brazil, Venezuela (BRITO A. C. & BITTENCOURT, 2018). The disease is less frequent in the Northern hemisphere, but cases have been described in Russia, Canada, Finland, Czech Republic, Romania, and Poland (PINDYCKA-PIASZCZYŃSKA et al., 2014). Since reporting of chromoblastomycosis is not compulsory, epidemiology is based on medical literature (QUEIRROZ-TELLES, 2015). Recent studies suggest an increasing number of cases of chromoblastomycosis, as like described for Verma et al., (2018), 70 patients over a period of 4 years India between 2013-2017; or 30 cases described in Taiwan (YANG et al., 2018), being *F. monophora* the mainly agent. From 2018, the cases reported in the literature are mostly associated to male have suffered injuries while working in agriculture. Among the identified species, the genus *Fonsecaea* is more frequent; and Brazil and China, are the countries with higher numbers of reported cases (Table 1). The number of cases rises among low-income populations, who live in the countryside since this mycosis is strictly related to agriculture (ANDRADE et al., 2019).

TABLE 1: Special cases of chromoblastomycoses related in the literature from 2018.

Year	*Gender*	*Age*	*Lesion site*	*Etiologic agent*	*Treatment*	*Status*	*Country*	*Reference*
2020	male	56	ankle	*Fonsecaea pedrosoi*	itraconazole and terbinafine	cured	China	Sheng et al., 2020
2020	male	45	hand	*Fonsecaea pedrosoi*	imiquimod	cured	Brazil	Belda et al., 2020
2020	male	43	hand	*Fonsecaea pedrosoi*	imiquimod	cured	Brazil	Belda et al., 2020
2020	male	39	wrist	*Fonsecaea pedrosoi*	imiquimod	cured	Brazil	Belda et al., 2020
2019	male	42	forehead, shoulder, and tibia	*Phialophora* spp.	fluoconazol	uncured	China	Qiu et al., 2019
2019	male	34	maxillary sinuses				India	Nandish; 2019
2019	male	52	pulmonary transplant				Spain	Calleja-Algarra et al., 2019
2019	male	86	arm				Brazil	Souza et al., 2019
2019	male	40	leg	*Rhinocladiella aquaspersa*	itraconazole and surgery	cured	Guatemala	Porras-López et al., 2019
2019	male	12	bones, cartilage, tongue and palatine tonsils		multiples	uncured	Pakistan	Khan et al., 2019
2019	male	54	leg				Brazil	Teles Filho; 2019
2019	male	60	knee			cured	India	Ittigi & Hegde, 2019
2019	male	52	leg		ALA-PDT	cured	China	Huang et al., 2019
2019	male	70	Fingers, wrist and elbow	*Cladophialophora carrioni*	Terbinafine and hyperthermia		China	Zheng et al., 2019
2019	male	34	knee	*Fonsecaea pedrosoi*	Terbinafine and hyperthermia	uncured	China	Zhang et al., 2019
2018	male	61	skin	*Fonsecaea spp.*	Heat monotherapy		Taiwan	Huang & Lan, 2018
2018	female	50	arm				India	Jaysree et al., 2018
2018	male	63	arm	*Fonsecaea pedrosoi*	Terbinafine and crioterapia, itraconazol	cured	México	Romero-Navarrete et al., 2018
2018	male	51	hand	*Rhinocladiella similis*	itraconazole and terbinafine	cured	Spain	Richarz, et al., 2018
2018	male	10	leg	*Phialophora verrucosa*			Costa Rica	Brenes et al., 2018
2018	male	71	leg	*Fonsecaea monophora*			Australia	Sideris & Ge., 2018
2018	male	33	leg	*Cladophialophora carrionii*	multiples	cured	USA	Turkowski et al., 2018
2018	female	13	leg	*Fonsecaea pedrosoi*	terbinafine and surergy		Maroc	Baline & Hali., 2018
2018	male	79	foot	*Phoma insulana*		uncured	Mexico	Hernández-Hernández et al., 2018
2018	male	59	leg		itraconazole and amphotericin B		Brazil	Resstel et al., 2018

The main risk factors associated with chromoblastomycosis are adult, male sex, rural work or outdoor activities, lack of protective footwear, gloves or clothing, and poor nutrition and hygiene habits (QUEIROZ-TELLES et al., 2017).

The etiological agents are mainly the fungi belonging to the genus *Phialophora*, *Rhinocladiella, Cladophialophora*, *Cyphellophora*, *Exophiala* and *Fonsecaea* (QUEIROZ-TELLES et al., 2015). *Rhinocladiella* genus, described in 1934 is associated to the cases reported in the American continent. In the present study, *R. mackenziei* was used as the agent of brain infections in healthy individuals associated with high mortality (ANDRADE et al., 2020; MORENO et al., 2018a). The *Phialophora* genus was introduced in 1915 by Medlar, also characterized by the presence of pigmented hyphae. The first species described was *P. verrucosa* isolated from a human skin lesion (HOOG et al., 1999), being the species of the genus more associated with cases of cutaneous chromoblastomycosis (RADOUANE et al., 2013). The first species of *Cladophialophora* genus was described in 1980 by Borelli, *C. ajelloi* Borelli, a cutaneous and subcutaneous chromoblastomycosis agent (QUEIRROZ-TELLES, 2017). *Cladophialophora carrionii* is one of the most frequent etiological agents of human chromoblastomycosis in dry and semi-arid climates; this species is characterized by moderate colony growth, with maximum growth temperature around 37°C and *C. bantiana* is among the main agents that affect the central nervous system (KANTARCIOGLU et al., 2017). *Exophiala* genus was formally described by J. W. Carmichael in 1966 and is composed of dimorphic melanized fungi, responsible for a spectrum of diseases, including phaeohyphomycoses, mycetomas, chromoblastomycosis and fungemia. *Exophiala jeanselmei* is the predominant species in these infections, followed by *E. dermatitidis* and *E. spinifera* (BADALI et al., 2010).

3.3 *Fonsecaea* GENUS

The first report of a species of *Fonsecaea* genus occurred in 1930 with the species *Hormodendrum pedrosoi*. The genus presents the second major chromoblastomycosis agent and is prevalent in tropical and humid subtropical climates now named *F. pedrosoi* (RODERICK, 2020). Esterre et al. (1996) reported 61.8% of cases of chromoblastomycosis of Madagascar present as etiological agent, a species of *Fonsecaea* genus. In Sri Lanka, 94% of the 71 cases of chromoblastomycosis were caused by these agents (RODERICK, 2020). In Gabon (equatorial Africa) all 64 reported cases of chromoblastomycosis

were caused by this genus. In Brazil, two studies reported high rates of chromoblastomycosis caused by *Fonsecaea* genus; 98% of 325 cases in the Amazon region reported in 1999 and 99% of cases reported in 2014 (DONG et al., 2014).

The most frequent agent of chromoblastomycosis is *F. pedrosoi* implicated in 99% of the cases reported (RODERICK, 2020). Although it is strictly related to the formation of muriform cells and post-traumatic corneal inflammation (CHAIDAROON et al., 2015). The second species with the highest frequency of isolation in clinical cases caused by this genus, is *F. monophora* (LIU et al., 2019). Also reported as an agent of brain infections invading the patient's central nervous system (THOMAS et al., 2018). Another species related to chromoblastomycosis is *F. nubica* (YOU et al., 2019). Morphologically it is very similar to *F. pedrosoi* and *F. monophora* and has been reported as an ethological agent of infection in several countries, such as China (FRANSISCA, et al., 2017), France (CATEAU et al., 2014) and southern African countries (SUN *et al*., 2012). The *F. pugnacius*, described in 2015 by Azevedo et al., presents particular characteristics to the genus, being the only species to cause lesions with muriform cells and abscesses in the brain in the same immunocompetent human patient.

Thus, the invasive potential differs significantly among species of *Fonsecaea* genus (GOMES et al., 2016). *Fonsecaea pedrosoi* and *F. nubica* are strictly associated with chromoblastomycosis and the formation of muriform cells. *Fonsecaea monophora* and *F. pugnacius* may also be associated with phaeohyphomycosis and have been reported in disseminated infections in the brain and other organs with hyphae in the tissue (VICENTE et al., 2017).

Although species of *Fonsecaea* genus are mainly related to infections in human hosts, other species of the genus have been related to the systemic infection with invasion of central nervous system in animal hosts (FENG & HOOG, 2017). The species *F. multimorphosa* was isolated from the brain of a cat that presented neurological disorders like loss of the coordination of the movements. Also considered an opportunistic species, since the animal host was in postoperative drug therapy (NAJAFZADEH et al., 2011b). Lethargic crab disease has been called this because of the symptoms it causes, leaving the animals slow and causing loss of muscle coordination. Since 1997, it has caused a high mortality rate of *Ucides cordatus*, an important crab species in the ecology and economy of the Brazilian coast (SCHMIDT, 2006). After the study, two species were associated with this disease, *Exophiala cancerae*, the primary agent of

infection and *F. brasiliensis* as opportunist (VICENTE et al., 2012).

Vicente et al. (2014) described environmental species for this genus: *F. erecta* and *F. minima*, isolated from environmental sources in Brazil. In the same study, *F. monophora, F. multimorphosa, F. brasiliensis* and *F. pedrosoi* were also isolated from organic matter in decomposition, indicating a possible mechanism of adaptation to the host. The *Fonsecaea* siblings are associated with several diseases, which are known to be caused by species that are morphologically very similar (GOMES et al., 2016), but the potential of infection seems to be polyphyletic and consequently differs between them. The pathogenic species present the compound life cycle and pathogenicity potential that justify the low environmental occurrence and the high frequency of infection in animal hosts (END & HOOG, 2017).

The black yeasts like *F. monophora* and *F. pugnacius* shared the same pathogenicity profile, being reported as a chromoblastomycosis agent and causal agent of primary brain infection in humans (LIU et al., 2019). The table 2 below shows morphological and genomic characteristics:

TABLE 2: General characteristics of the *Fonsecaea* species associated to disseminated infection.

	F. monophora	***F. pugnacius***
Morphology	Conidiophores erect, un-branched or branched with hyphae or slightly darker.	Conidiophores erect, olivaceous brown, apically densely branched; some- times slightly differentiated, loosely branched.
Colonies	Restricted, spreading moderately, lanose to velvety, olivaceous to black, reverse black.	Restricted, compact, circular, olivaceous gray; reverse olivaceous black.
Hyphae	Septate, pale olivaceous.	Septate, smooth walled, pale olivaceous.
Optimal growth	At 27 to 33°C.	At 27 to 33°C.
Genome Size	34,21 Mbp	34,8 Mbp
GC%	52,12	52
tRNA	32	35
Proteins	11,984	12,217

The *F. monophora* was isolated in South America in 19 36, from chromoblastomycosis case described by Hoog, G.S; Vicente, V.A. & D. Attili (2004). While, *F. pugnacius* was identified in a chronic case of chromoblastomycosis where

caused secondary cerebritis by dissemination to the brain, despite immunocompetent patient (AZEVEDO et al., 2015).

Micro and macromorphologically, the sibling species are really close showing conidiophores erect, hyphae are septate and similar colors (Fig. 1 A-D) (HOOG et al., 2004; AZEVEDO et al., 2015). Since 2004, with the molecular information of *F. monophora* (HOOG et al., 2004), this species also emerged as like a chromoblastomycosis agent, being the predominant agent in southern China (FRANSISCA et al., 2017) and new clinical cases of have been reported every year (LABEL et al., 2018; STOKES et al., 2017; HELBIG et al., 2018; DOBIAS et al., 2018). The increase in reported clinical cases associated with this species signals a possible misunderstanding in the identification of the etiologic agents of chromoblastomycosis, because *F. pedrosoi* reported in most of the previously published reports has significant morphological similarity with *F. monophora* (PINDYCKA-PIASZCZYŃSKA et al., 2014).

In the histopathology analysis of infection by *F. pugnacius* case, the cells observed in skin and the brain tissues are very different, muriform cells are produced in skin, but hyphae are produced in brain, indicating a change in cell conformation to an invasive morphology (Fig. 1 E-F). This type of dissemination and the apparent conversion to another invasive morphology have not been observed in other species, suggesting one ability of this new species. Although the mechanism of infection of these fungi is unknown, especially in immunocompetent hosts, the neuroinvasive infection caused by *F. pugnacius* appears to be different from that caused by *F. monophora*, where primary human brain infection are reported to be caused by inhalation and occurring primarily in immunocompromised hosts (AZEVEDO et al., 2015).

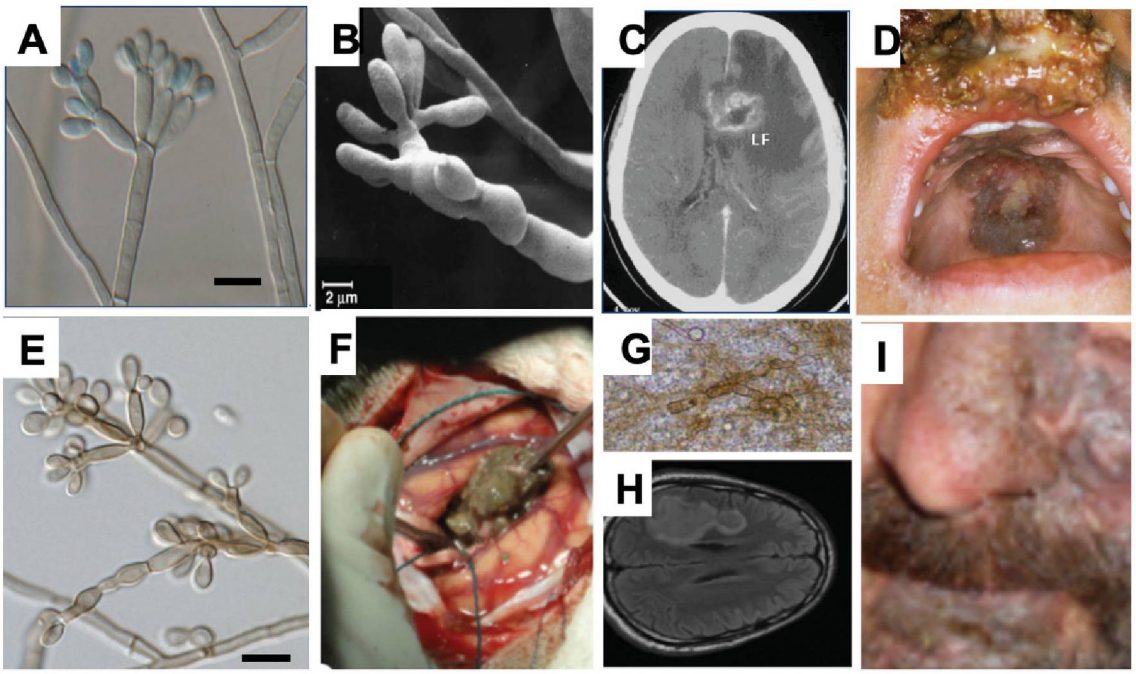

FIGURE 1: Morphological and clinical aspects of *F. monophora* CBS 102225 and *F. pugnacius* CBS 139214. *F. monophora:* A) Conidial apparatus bearing short conidial chains; B) Conidiogenous cell with conidia; C and D invasive brain human infection (HOOG et al., 2004; SURASH, et al., 2005). *F. pugnacius:* E) Conidiophores and conidia; F) biopsy of brain infection; G) Fungal cells observed in the abscess walls. H and I) Clinical aspects of disseminate infection *(*AZEVEDO et al., 2015).

Fonsecaea monophora presents a variable clinical spectrum, showing great versatility and ample adaptability, being a chromoblastomycosis agent with formation of muriform cells and agent of primary brain infection. Although it is more frequent in immunosuppressed patients, it can affect patients with intact immunity (NAJAFZADEH et al., 2009). While, *F. pugnacius* presented a new ability to disseminate causing fatality in immunocompetent individuals (AZEVEDO et al., 2015).

Phaeohyphomycosis caused by *F. monophora* is a rare disease, as eleven cases have been reported in the world literature. The most frequent manifestation with lesion restricted to a single lobe (DOBIAS et al., 2018), but the associations with infection are varied: Four patients was type 2 diabetes mellitus (DOYMAZ et al. 2015; STOKES et al. 2017; SURASH et al. 2005; VARGHESE et al. 2016), two patients were infected by solid organs transplantation in two patients (KOO et al. 2010; TAKEI et al. 2007), one was HIV serum positive (BAGLA et al. 2016), traumatic inoculation of the fungus (NOBREGA et al. 2003) and otitis media (SURASH et al., 2005).

This information has been directing virulence tests, like as the study by JIANG et al., (2018), the melanin pathway was inhibited, and the immunity response was analyzed. GARCIA SILVA-BAILÃO et al., (2018) amplified the knowledge about the biology and pathogenicity of these organisms, as the unique apparatus for the regulation of zinc and copper homeostasis. Recently reports showed the new diagnostic techniques, like the specific primers based on the *CBF5* gene developed for *F. pedrosoi*, *F. monophora*, *F. nubica*, and *F. pugnacius* and a probe designed for *F. pugnacius* based on ITS sequences (SCHNEIDER et al., 2019).

3.4 *Fonsecaea* SPECIES: INSIGHTS IN GENOMICS ANALYSIS, VIRULENCE AND PATHOGENICITY

The application of new sequencing technologies to elucidate the genetic of pathogenicity and niche adaptation in black yeast like started when the first whole-genome sequence of the *Exophiala dermatitidis* was reported by the Broad Institute (http://www.broadinstitute.org/annotation/genome/Black_Yeasts/MultiHome.html). Since that publication, sequencing and analysis of an additional 37 genomes and transcriptomes, produced an avalanche of data for comparative genomics (MORENO et al .2018b). These genomes comprises species that display a wide diversity of lifestyles, including the human opportunists involved in disseminated and neurotropic infections *C. bantiana*, *F. monophora*, *E. dermatitidis,* and *R. mackenziei*; the etiologic agents of chromoblastomycosis *C. carrionii* and *F. pedrosoi* and their environmental counterparts *C. yegresii* and *F. erecta*, respectively; the species associated with the degradation of toxic hydrocarbons *E. oligosperma, E. xenobiotica, E. spinifera, C. immunda* and *C. psammophila*; the ant-associated *Phialophora attae;* and several saprobic species occasionally reported causing mild infections. These genomes have been produced using short-read approaches, based on the Illumina, Ion Torrent and/or 454 instruments (MORENO et al 2018b).

In recently studies on comparative genomics analyses reporting ecological preferences of *Chaetothyriales* (TEIXEIRA et al. 2017; VICENTE et al. 2017) has been reported general characteristics, phylogenomic relationships, transposable elements, sex-related genes, protein family evolution, genes related to protein degradation (MEROPS), carbohydrate-active enzymes

(CAZymes), melanin synthesis and secondary metabolism were investigated and compared between species.

In the study published by Teixeira et al 2017, the authors demonstrated that in the bantiana-clade contained the highest number of predicted genes (12,817 on average) as well as larger genomes. Additionally, they identified a reduction of carbohydrate degrading enzymes, specifically many of the Glycosyl Hydrolase GH) class, while most of the Pectin Lyase (PL) genes were lost in etiological agents of chromoblastomycosis and phaeohyphomycosis. An expansion was found in protein degrading peptidase enzyme families S12 (serine-type D-Ala-D-Ala carboxypeptidases) and M38 (isoaspartyl dipeptidases). Based on genomic information, a wide range of abilities of melanin biosynthesis was revealed; genes related to metabolically distinct DHN, DOPA and pyomelanin pathways were identified. The *MAT* (*MA*ting *T*ype) locus and other sex-related genes were recognized in all 23 melanized fungi used in this study.

Furthermore, fungi exhibit a wide diversity of reproductive modes involving sexual, asexual, and parasexual cycles responsible by their recombination and consequently reflect direct to the virulence traits, and adaptations in new ecological niches. In this context to know the genes related to this process is important to understand the evolutionary steps of a specific group. According to Teixeira et al. 2017, members of the asexual genera *Fonsecaea* and *Cladophialophora* appear to be heterothallic with a single copy of either *MAT-1-1* or *MAT-1-2* in each individual. All *Capronia* species are homothallic as both *MAT1-1* and *MAT1-2* genes were found in each single genome. The authors demonstrated that the genomic synteny of the *MAT*-locus flanking genes (SLA2-APN2-COX13) is not conserved in melanized fungi as commonly observed in *Eurotiomycetes*, indicating a unique genomic context for *MAT* in those species. The heterokaryon (het) genes expansion associated with the low selective pressure at the *MAT*-locus suggests that a parasexual cycle may play an important role in generating diversity among those fungi.

A comparative genomic analysis of environmental and pathogenic siblings of *Fonsecaea* and *Cladophialophora* was undertaken by Vicente et al, in 2017, including *de novo* assembly of *F. erecta* from plant material. The genome size of *Fonsecaea* species varied between 33.39 and 35.23 Mb, and the core genomes of those species comprises around 70%. Expansions of protein domains such as glyoxalases and peptidases suggested ability for pathogenicity in clinical agents, while the use of nitrogen and degradation of phenolic compounds was enriched in environmental species. The similarity of carbohydrate-active vs. protein-degrading

enzymes associated with the occurrence of virulence factors suggested a general tolerance to extreme conditions, which might explain the opportunistic tendency of *Fonsecaea* sibling species.

According to Moreno et al (2018a), apparently there is an association between genome size and ecological preferences. In the Bantiana-clade a potential explanation for the larger genomes in many species is the striking abundance of the protein families short chain dehydrogenase, CYP P450, aldehyde dehydrogenase, sugar transporters, and membrane transporter proteins.

Vicente et al. (2017) also analyzed common genes among *Fonsecaea* and *Cladophialophora* species related to subcutaneous and disseminate infections showing 43 gene clusters shared by agents of systemic infection and 32 gene clusters related to (sub)cutaneous infection. Annotation of these clusters in *Fonsecaea* species revealed 33 domains related to disseminate infection and 24 domains related to subcutaneous infection.

Moreno et al. (2018a) reported a study based on genomic understanding of an infectious brain disease caused by *Rhinocladiella mackenziei* strains isolated from patients in Saudi Arabia and Qatar. The authors showed the intra-species variation and genetic signatures to uncover the genomic basis of the pathogenesis and potential niche adaptations. Comparative genomics with other filamentous ascomycetes revealed a diverse arsenal of genes likely engaged in pathogenicity, such as the degradation of aromatic compounds and iron acquisition. In addition, the authors indicated that intracellular accumulation of trehalose and choline suggests possible adaptations to the conditions of arid climate region. Gene composition and metabolic potential indicate extremotolerance and hydrocarbon assimilation in *R. mackenzei*. Specifically, protein family contractions were found, including short-chain dehydrogenase/reductase SDR, the cytochrome P450 (E-class) and the G-protein beta WD-40 repeat.

According to Vicente et al. (2017,) the primary fungal pathogens attempt to disrupt host cell homeostasis while avoiding and/or suppressing host recognition. In opportunists these mechanisms are not sophisticated and probably have emerged due to flexibility in nutrient acquisition (DICKMAN & FIGUEIREDO, 2011) and extremotolerance (MORENO et al., 2017). Casadevall (2007) suggested that this unfocused virulence explains the “dual use” determinants in unexpected agents of disease.

Virulence was tested in the *Galleria mellonella* model and immunological assays were performed in order to support this hypothesis. Larvae infected by environmental *F. erecta* had a lower survival. Fungal macrophage murine co-culture

showed that *F. erecta* induced high levels of TNF-α contributing to macrophage activation that could increase the ability to control intracellular fungal growth although hyphal death was not observed, suggesting a higher level of extremotolerance of environmental species (VICENTE et al. 2017). Futhermore, Fornari et al. in 2018, demonstrated by immunologic assay in BALB/c mice, that the high virulence of saprobic species in animal models was subsequently controlled via host higher immune response.

Moreno et al (2018b) called attention about the next stage of black yeasts like studies named as post-genomic era, translating knowledge from DNA to transcriptome and proteome analyses, in order to elucidate the transcriptional response of black yeasts to stressors such as temperature, pH, radiation, and toxicity. The first reported of trancriptome analisys was in *Exophiala dermatitidis*, which is assumed as a model for melanized agents of the human and animal infection. The transcriptome of an albino strain of *F. monophora* was compared with its melanized parent by Li et al., (2016) identifying 2283 differentially expressed genes between the genotypes. Extensive down-regulation of key genes in the DHN pathway was shown in the albino mutant, which was also more susceptible to low pH, high UV radiation, and oxidative stress. Acording to the comparative genomic analyses the three pathways for melanin production were detected in *Fonsecaea* sampling species: via 1,8-dihydroxynaphthalen (DHN), via 3,4-dihydroxyphenylalanine (DOPA), and via l-tyrosine degradation (Vicente et al. 2017).

Although, this technology was used to describe genomes it has being encouraged as a good tool in order to elucidate the chromosome groups. Polyploidy, aneuploidy, and gross chromosome rearrangements have been recognized as drivers of genetic diversity in pathogenic and non-pathogenic fungi (MONERAWELA & BOND 2017; HARARI et al. 2018; RUIZ et al. 2019). In pathogenic yeasts, such as *C. albicans*, mechanisms for ploidy shifts and chromosome rearrangements have been described, and their importance for adaptation to environmental stresses and host niches, as well as for developing resistance to antifungal drugs has been identified (WERTHEIMER et al., 2016).

Many attempts have been made to study the chromosomes of fungi, but a major problem is that fungal nucleus is so small. Fungal chromosomes are at the lowest resolution of light microscopy; thus, few attempts to visualize fungal chromosomes have been successful. The number of chromosomes per nucleus, estimated by conventional light visualization and stained with standard dyes like Giemsa or aceto-orceine, usually does not exceed ten (MAHMOUD & TAGA, 2012).

A method developed in the late 1980s called 'germ tube burst' enables the discharge of condensed chromosomes from the hyphal cell and their spread on the surface of a slide. This more accurate method usually gives better resolution of chromosomes. It was used with conventional light microscopy dyes as well as in fluorescent microscopy or for in situ hybridization. Another methodology by using the pulse field gel electrophoresis (PFGE) that make possible the separation of the chromosomes on the gel enables the determination of their number and estimation of genome size. Both methods can be associated allowing the complex analysis of fungal genomes (MEHRABI & TAGA, et al. 2017).

It was developed in 1984 with focus on supply the limitations of traditional fungal cytogenetics. During the past three decades, PFGE has been extensively used for fungal karyotyping, particularly because it enables the visualization of small chromosomes and is independent of meiosis (MEHRABI & TAGA, 2017). Pulsed field gel electrophoresis (PFGE), or electrophoretic karyotyping, separates chromosomal-sized pieces of DNA in agarose gels where the orientation of the electric field is periodically altered. This technique has revealed that many fungi have a high degree of chromosomal length polymorphisms (WIELOCH, 2006).

The PFGE has been used to estimate chromosome numbers and sizes of many fungal plant-pathogen genomes. Prior to the advent of next-generation sequencing, karyotype profiling of fungal chromosomes was the only way to determine chromosome numbers and to estimate overall genome size (MEHRABIET al. 2017), such as the electrophoretic and cytological analyses of the *Z. tritici* strain IPO323 revealed that It had 20 chromosomes with a genome size of approximately 40 Mb, after complete genome analysis, it was confirmed a total of 21 chromosomes and 39.68 Mb genome size (GOODWIN et al., 2011).

This technique can also be used to follow inheritance of chromosomal length polymorphisms and shows that in some fungi novel-sized chromosomes are produced during meiosis (HOWLETT ,1997). Intact chromosomes of 19 clinical isolates of *Histoplasma capsulatum* was obtained in Argentina, Mexico and Guatemala and Panama, were analyzed using pulsed-field gel electrophoresis showing 5-7 bands, ranging from 1.3 to 10 Mbp in size (CANTEROS et al., 2005).

The PacBio RS II instrument (Pacific Biosciences, Menlo Park, CA) represents the third-generation sequencer able to sequence a single molecule DNA in real-time without amplification. PacBio sequencing technology is novel and unique, enabling the direct observation of DNA synthesis by DNA polymerase confers advantages compared to other sequencing technologies, such as long read lengths, high consensus accuracy and simultaneous capability of epigenetic

characterization (NAKANO et al. 2017). The PacBio assembly can produced 100 times longer contigs with 100 times smaller number of gaps compared to the other sequencing platafform. Some regions are difficult to reconstruct by short-read sequencers such as the chromosome size, shape and number in genome fungal (RHOADS & AU 2015).

Schultzhaus et al. (2019) used the PacBio single-molecule real-time (SMRT) sequencing to assist with understanding the molecular basis of its uncommon morphological and metabolic characteristics of the melanized dimorphic fungus *Exophiala lecanii-corni*, with presents the capability of degrading several volatile organic compounds.

De novo genome assembly is one of the main applications of PacBio sequencing because long reads can provide large scaffolds. PacBio long reads overcome many limitations of genome assembly using short-read approaches, such as the presence of highly-repetitive genomic regions and represents an option to establish new insights in Chaetothyriales, e.g. closing gaps in draft genomes. Zhang et al. in 2012 compared this gap closure method with Sanger sequencing for 362 gaps ranging from 500 bp to 5 kb from 16 diverse genomes demonstrating this applicability of the methods.

In summary the use of technologies, such as genomics, transcriptomics, and proteomics, to acess the genetic information, is now a reality, and an enormous amount of data has already been generated in Chaetothyriales. According to Moreno et al. (2018a), the genomes sequence of a wide variety of species is only a first step to shed light on adaptive processes, including shifts to pathogenicity. The next stage is translating knowledge from DNA to transcriptome and proteome analyses. Although the molecular machinery of pathogenicity is far from being elucidated, important indications have been obtained. Moreover, other initiatives, such as the use of in vivo models for mimicking the state of infection must be adopted.

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CHAPTER II

Comparative Genomics of Sibling Species of *Fonsecaea* Associated with Human Chromoblastomycosis

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Comparative Genomics of Sibling Species of *Fonsecaea* Associated with Human Chromoblastomycosis

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Fonsecaea and *Cladophialophora* are genera of black yeast-like fungi harboring agents of a mutilating implantation disease in humans, along with strictly environmental species. The current hypothesis suggests that those species reside in somewhat adverse microhabitats, and pathogenic siblings share virulence factors enabling survival in mammal tissue after coincidental inoculation driven by pathogenic adaptation. A comparative genomic analysis of environmental and pathogenic siblings of *Fonsecaea* and *Cladophialophora* was undertaken, including *de novo* assembly of *F. erecta* from plant material. The genome size of *Fonsecaea* species varied between 33.39 and 35.23 Mb, and the core genomes of those species comprises almost 70% of the genes. Expansions of protein domains such as glyoxalases and peptidases suggested ability for pathogenicity in clinical agents, while the use of nitrogen and degradation of phenolic compounds was enriched in environmental species. The similarity of carbohydrate-active vs. protein-degrading enzymes associated with the occurrence of virulence factors suggested a general tolerance to extreme conditions, which might explain the opportunistic tendency of *Fonsecaea* sibling species. Virulence was tested in the *Galleria mellonella* model and immunological assays were performed in order to support this hypothesis. Larvae infected by environmental *F. erecta* had a lower survival. Fungal macrophage murine co-culture showed that *F. erecta* induced high levels of TNF-α contributing to macrophage activation that could increase the ability to control intracellular fungal growth although hyphal death was not observed, suggesting a higher level of extremotolerance of environmental species.

Keywords: *Fonsecaea* species, black yeast, genomics, chromoblastomycosis, comparative genomics, *Fonsecaea erecta*

INTRODUCTION

Melanized fungi belonging to the order Chaetothyriales are clinically relevant as agents of a gamut of diseases in humans and animals, varying in severity from superficial to systemic and fatal infections (De Hoog et al., 2004; Badali et al., 2008; Seyedmousavi et al., 2011). A large number of species have complex life cycles, indicating dynamic niches or vectored transmission (Sudhadham et al., 2008). In the environment, they occupy adverse micro-habitats, which is probably stimulated by their low competitive ability toward co-occurring microorganisms, judged from the fact that their isolation is enhanced significantly by the use of selective methods (Vicente et al., 2014). Many species of Chaetothyriales cause implantation diseases from an environmental source. One of the common disorders is chromoblastomycosis, a mutilating and recalcitrant skin disease eventually leading to emerging eruptions. Fungal cells in host tissue provoke as an inflammatory granulomatous disease (De Hoog et al., 2007; de Azevedo et al., 2015a; Queiroz-Telles, 2015). Agents of chromoblastomycosis are traumatically inoculated from environmental sources such as plant thorns or wooden splinters carrying the respective opportunist (Salgado et al., 2004; De Hoog et al., 2007; Vicente et al., 2014). Those species are mainly found in *Fonsecaea* and *Cladophialophora*, of which *F. pedrosoi, F. monophora*, and *Cladophialophora carrionii* are recurrently recovered from patients in tropical and semi-arid climate zones, respectively around the globe (Xi et al., 2009; Queiroz-Telles, 2015). Recently, less common agents were described, such as *F. nubica* (Najafzadeh et al., 2010), *F. pugnacius* (de Azevedo et al., 2015b), and *C. samoensis* (Badali et al., 2010). The genera *Fonsecaea* and *Cladophialophora* are morphologically classified by differences in their conidial apparatus; however, DNA polymorphisms suggest that both genera are polyphyletic (De Hoog et al., 2007), as they are distributed within "bantiana-clade" and "carrionii-clade" groups of the family Herpotrichiellaceae (Chaetothyriales) (Vicente et al., 2014; de Azevedo et al., 2015b).

Closely related species of *Fonsecaea* differ significantly in their ecology and ability to cause infection in humans and animals (Vicente et al., 2014); virulence genes seem to be unequally distributed among members of the bantiana-clade. *Fonsecaea pedrosoi* and *F. nubica* are strictly associated to chromoblastomycosis, while *F. monophora* also causes primary brain disease (Surash et al., 2005; Takei et al., 2007; Koo et al., 2010). *Fonsecaea multimorphosa* and *F. brasiliensis* were isolated from disseminated infections in animal hosts (Najafzadeh et al., 2011; Vicente et al., 2012) and the environmental species *F. erecta* and *F. minima* were described from plants and no report from clinical cases (Vicente et al., 2014) has as yet been published.

Therefore, the central question of the present study concerns the difference in infectious potential between closely related members of *Fonsecaea*. Agents of chromoblastomycosis upon tissue invasion show dimorphism to muriform cells, while this behavior is not known from most plant debris-inhabiting siblings (Queiroz-Telles, 2015). Comparative genomic analysis of *Fonsecaea* pathogenic and non-pathogenic siblings was applied to highlight genes involved in fungal adaptation from a plant debris- to an animal-associated lifestyle. To this aim, a *de novo* assembly of *F. erecta* and recently published black yeast genomes (Bombassaro et al., 2016; Costa et al., 2016; Leão et al., 2017; Teixeira et al., 2017) was included in the analysis.

MATERIALS AND METHODS

Genomic DNA Extraction, Sequencing, and *de Novo* Assembly of *Fonsecaea erecta* CBS 125763^T

A large-scale DNA extraction was conducted based on a method as described by Vicente et al. (2008). The strains were grown in Sabouraud broth for 7 days. The DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method based and phenol-chloroform/isoamyl alcohol. Total DNA was purified with the Microbial DNA UltraClean™ kit. The genome was sequenced on MiSeq (Illumina™) sequencer using paired-end and mate-paired libraries and on Ion Proton (Thermo Fisher Scientific™) sequencer using single-end approaches. The library construction was done with Ion Plus Fragment Library Kit (Thermo Fisher Scientific™) and Nextera XT (Illumina™) following the manufacturer instructions. The read quality analysis was performed with FastQC and reads with quality below PHRED20 were removed (Andrews, 2016). The reads were assembled *de novo* using SPAdes v3.6.2 (Bankevich et al., 2012). The gap closure was performed with FGAP (Piro et al., 2014) and scaffolding with SSPACE (Boetzer et al., 2010). The mitochondrial genomes were assembled in *Fonsecaea* species by extracting reads using the complete mtDNA of *Exophiala dermatitidis* as reference. The reads were mapped using Bowtie2 (Langmead and Salzberg, 2012) and the mapped reads assembled with SPAdes v3.6.2 (Bankevich et al., 2012).

Gene Prediction and Annotation

Protein-coding genes were predicted with GeneMark-ES v4.39 (Besemer, 2001). The automatic annotation was done by RAFTS3 (Vialle et al., 2016) best hits comparison with self- score cutoff of 0.5 using a black yeast protein database available on (www.broadinstitute.org/annotation/genome/Black_Yeasts/). Protein domain families and functional annotation was accessed using InterProScan5 (Quevillon et al., 2005). The tRNAs annotation used the ARAGORN software (Laslett and Canback, 2004). Putative enzymes and peptidases coding genes using CAZY (Cantarel et al., 2009) and peptidases coding genes using MEROPS database (Rawlings et al., 2015) and putative

pathogen host interaction genes using PHI base (Winnenburg et al., 2007). Pathogen Host interacting (PHI) partners were identified by subjecting the predicted proteomes to BLASTp against the PHI database v4.2 with an *E*-value threshold of 10–5 with best hits.

Comparative Genomic Analysis of the Genus *Fonsecaea*

We compared the genome of *F. erecta* CBS 125763^T to 5 *Fonsecaea* species, including *F. monophora*, CBS 269.37^T (Bombassaro et al., 2016), *F. nubica* CBS 269.64^T (Costa et al., 2016), *F. multimorphosa* CBS 980.96^T (Leão et al., 2017), *Fonsecaea multimorphosa* CBS 102226 (Teixeira et al., 2017), and *F. pedrosoi* CBS 271.37^T (Teixeira et al., 2017) in addition to other 6 black yeast-like fungi belonging to the order Chaetothyriales (Teixeira et al., 2017): *Cladophialophora carrionii* CBS 160.54, *Cladophialophora yegresii, Capronia semiimmersa, Cladophialophora bantiana, Cladophialophora psammophila, Cladophialophora immunda,* and *Rhinocladiella mackenziei* (Table 2).

Protein sequences were compared using an all-vs.-all similarity search and self-score cutoff of 0.5 using RAFTS3 (Vialle et al., 2016). The clustering was done when at least one protein was shared amidst clusters. After the clustering verification step was done K-means and the cluster vectors where split into new clusters using the ratio of the cluster size and the number of organisms present in the analysis. For the resulting clusters it was calculated a centroid for each vector and chosen the best gene that represents each cluster based on the shortest distance. For both K-means and centroid analysis, a vectorial representation for the genes was created based on sparse *k*-mers sequences. A final clustering step was done using RAFTS all-vs.-all similarity searches with self-score of 90 (Vialle et al., 2016). The amino-acid sequences of each family were aligned with Muscle (Edgar, 2004) and poorly aligned regions were automatically removed using GBLOCKS (Talavera and Castresana, 2007). A maximum likelihood tree was done using PHYML (Guindon et al., 2005) and 1,000 bootstraps were used to infer branch support.

$$r_{rp} = \frac{M_1 - M_0}{S_n}\sqrt{\frac{n_1 n_0}{n^2}}$$

FIGURE 1: The point-biserial correlation coefficient: measure of the relationship between a continuous and a binary variable. For each protein of the 26 analyzed genomes, 0 and 1 scores correspond to the presence or absence of a protein of the binary variable, respectively. M1 is the mean of the presence of proteins and M0 is the mean of the missing proteins. The value "n" represents the total number of the proteins, where n1 is the total of proteins present and n0 are the total of the missing proteins. Sn is the standard deviation of the continuous variable.

Genome Expansions and Contractions Based on Functional Domains

To identify functional expansions and contractions, InterPro domains were predicted using InterProScan5 (Quevillon et al., 2005) for 12 strains of black yeast species: 6 *Fonsecaea* species, 5 *Cladophialophora* species and 1 *Coniosporium apollinis* as outgroup (Table 2). Gene family evolution was estimated with CAFE version 3.0 (De Bie et al., 2006) using significance family-wide *p*-values threshold of <0.05 and VITERBI $p < 0.001$. To search for BIRTH (λ) values we run the program with the "-s" option. Two files were used as input in CAFE analyses: a table containing organism number of copies of each InterPro domain and an ultrametric tree.

Prediction of Genes Related to Pathogenicity

In order to research genes related to pathogenicity through analysis of the core, clusters and perform a correlation analysis, initially were rescued the 22 yeast genomes available at Broad Institute (http://www.broadinstitute.org/annotation/genome/ Black_Yeasts/GenomesInde) and including the *Fonsecaea* sibling associated to (sub)cutaneous and systemic infection, totaling 26 genomes. They were all (re) annotated using toolbox RAFTS3 (Vialle et al., 2016). The next step was to vectorize and cluster each gene, which generated 28,355 gene clusters equal or bigger the 50% of identity between them. This analysis was sized in an array with

26 rows × 28,355 columns, the rows being the organisms and the columns all the genes of all clustered organisms.

To perform the correlation analysis between the clusters using Point-biserial correlation to each of 28,355 clusters, we selected a set of genes that was used as reference to access a set of already known pathogenic genes, being them: Cell Division Control Protein 42 (KIV82855.1), Cytochrome P450 (KIW97819.1), Thioredoxin (XP_013289847.1), HSP60-like protein (KIW92920.1), HSP90-like protein (AYO21_00238), Homogentisate 1,2-dioxygenase (KIW31930.1), and two hypothetical proteins (AYO21_05248 and KIW22607.1) which were present in the same clusters of paraoxonases.

The correlations of each cluster with the frequency of these pathogenic set genes in the organisms were calculated according to formula (Figure 1), which was filtered in 1,803 clusters of genes. Analyzing the gene families within organism related to systemic infection (*F. multimorphosa, C. bantiana, F. monophora,* and *R. mackenziei*) and subcutaneous infection (*F. pedrosoi, C. carrionii, F. monophora,* and *F. nubica*) only 280 showed positive correlation above 80% with 5.5×10^{-7} of max *e*-value.

Virulence Test of *Fonsecaea* Sibling Species Using Galleria mellonella Larvae As a Model

Fungal Strains, Growth Condition, and Inoculum Preparation

The strains *F. pedrosoi* ATCC 46428, *F. pedrosoi* CBS 271.37, *F. erecta* CBS 125763 and *F. monophora* CBS 102248 were selected for this study. The yeast strains were grown on Sabouraud Glucose Agar (SGA; Himedia, Mumbai, India) at 28°C for 7 days, transferred to Potato Dextrose Broth (PDB; Himedia) and incubated under agitation (150 rpm) at 37°C. After 5 days, the cultures were allowed to settle in order to decant the larger particles such as hyphae and conidia. Fungal cells were separated by filtration through a 40 μm cell strainer, (BD) washed with PBS 1× three times. Finally, the cells were re-suspended at 1×10^6 cells/mL for *F. pedrosoi* ATCC 46428, *F. pedrosoi* CBS 271.37, and *F. erecta* CBS 125763, or at 1×10^5 cells/mL for *F. monophora* CBS 102248.

Larvae Selection and Infection

Galleria mellonella larvae were selected using as criteria similar size and weight ranging 0.10–0.15 (g). For the survival experiments, each group consisted of 20 larvae. The selected larvae were inoculated by injecting 10 μL of the different inoculum in the last left pro-leg with a Hamilton syringe (0.75 mm diameter needle) according to Fuchs et al. (2010). The control group was inoculated with PBS and the same number of larvae. The following control groups were used in the experiment. The first group included the larvae that received 10 μL of PBS to monitoring survival mortality related to trauma. A second group of larvae (SHAM) received no injection and no injury. All larvae were placed in sterile Petri dishes and kept in the dark at 37°C. Mortality was monitored once per day. The death of the larvae was assessed by the lack of movement, no response to stimulation and discoloration of the cuticle. Melanization was checked every 24 h with a NIKON D3100 camera and images were analyzed. Survival curves were plotted and statistical analyses were performed using the Log-rank (Mantel-Cox) test with Graph Pad Prism software. Statistical differences were set at $p < 0.05$.

Histopathology

Larvae were fixed by immersion in Carnoy (60% methanol, 30% chloroform and 10% acetic acid) 7 days after infection. After 48 h the larvae were immersed in 70% ethanol. Then the sections were then embedded in paraffin wax, sectioned and stained with Periodic Acid-Schiff (PAS) for microscopic examination. The photomicrographs were obtained from Olympus BX41 microscope coupled with digital camera Olympus SC30.

Fungal Macrophage Co-culture

The strains *F. pedrosoi* CBS 271.37 and *F. erecta* CBS 125763 were cultivated on Sabouraud Glucose Agar (SGA, Himedia) supplemented with 100 mg/L^{-1} chloramphenicol at 37°C. To obtain purified conidia and hyphae, fungi propagules were grown in PDB supplemented with 100 mg/L^{-1} chloramphenicol, in a rotary shaker (120 rpm) at 30°C. Fungal purification was performed according to Siqueira et al. (2017). Briefly, 15 days suspension containing conidia and hyphal fragments was subjected to successive filtrations through 70 μm and 40 μm cell strainers (BD). Hyphae retained on the 40 μm cell strainer were re-suspended in phosphate buffered saline (PBS), centrifuged at 1,000 × g and re-suspended in PBS. This process was repeated twice. Ninety-eight percent of 98% of this suspension consisted of hyphae. The 40 μm cell strainer filtrate containing conidia and small hyphal fragments was subjected to a filtration through 14 μm filter paper (J. Prolab, Brazil), washed twice in PBS, and recovered by centrifugation at 3,000 × g. This fraction contained conidia more than 98% pure.

Macrophage infection assays were adapted from Hayakawa et al. (2006), Palmeira et al. (2008) and Siqueira et al. (2017). Mouse macrophages (J774 cell line) were plated in DMEM (Dulbecco's Modified Eagle's medium, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and infected with conidia or hyphae of the *Fonsecaea* species at a multiplicity of infection (MOI) of 1. After 3 h of infection, non-phagocytized fungi were washed, fresh medium was added and co-culture was allowed to proceed for 24 h. For fungal burden determination (number of fungal cells in macrophages), macrophages were lysed with 0.05% SDS solution.

Intracellular fungi were quantified by plating serial dilutions of cell lysates onto SDA medium, supplemented with 100 mg/L^{-1} chloramphenicol and cultivated at 30°C for 7 days. Fungal burden was then measured by counting fungi colony-forming units (CFU). The cell culture supernatants were used to determine tumor necrosis factor-α (TNF-α) levels by enzyme-linked immunosorbent assay (ELISA), in accordance with manufacture's (eBioscience) instructions. As positive control to TNF-α production, 500 ng/mL LPS (*Escherichia coli* serotype 0111:B, Sigma-Aldrich) was used. Results were expressed as number of CFU or pg/mL of cytokine ± standard deviation (SD).

Mice Infection

BALB/c mice were maintained under standard laboratory conditions. Mice (10–12 weeks old males) were inoculated by injecting 50 μL (per hind footpad) of PBS containing 1×10^6 *F. pedrosoi* or *F. erecta* propagules obtained by mixing the purified hyphae and conidia in the proportion of 3:1, respectively. Five animals per group were euthanized with CO_2 in an appropriate chamber at 7 and 14 days post-infection. The mice footpad were photographed, removed, weighed and thereafter homogenized in tubes with steel beads on a Precellys homogenizer. For fungal burden determination, homogenized tissue were diluted and plated as above mentioned. Results were expressed as number of CFU ± standard error of mean (SEM) per gram of fresh tissue. Cytokine production was measured from homogenized tissue obtained from infected and non-infected animals (healthy) by ELISA. The cytokines interleukin-1β (IL- 1β), TNF-α, interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1/Ccl2) were measured with kits purchased from eBioscience and used according the manufacturer's instructions. Results were expressed as pg of cytokine ± standard error of mean (SEM) per 100 milligrams of tissue. All animal experimentation in this study were approved by the Federal University of Paraná Ethics Committee (approval certificate 1002) and performed according to the Committee's recommendations.

RESULTS

De Novo Assembly of *Fonsecaea erecta* and Genome Contents of *Fonsecaea* Siblings

Whole genome sequencing of *Fonsecaea erecta* CBS 125763^T was performed using Illumina Hiseq 2000 and yielded 1,534,038 paired-end reads with average insert size of 1 Kb ± 1 Kb and 3,133 mate-paired reads with average insert size of 5 Kb ± 4 Kb. To increase sequence coverage, two additional Ion Torrent shotgun libraries were sequenced generating 5.4 Gb and 25,219,375 single reads. The final high quality draft genome of *Fonsecaea erecta* comprised 57 scaffolds. The genome size was estimated to be 34.75 Mb, with average coverage of 60X and G+C content of 53%. Protein coding regions account for 18,279,031 bp, corresponding to 12,327 genes. A total of 12,090 proteins encoding genes, one rRNA multi-copy segment and 30 tRNA genes were predicted (Table 1).

TABLE 1: *Fonsecaea erecta* genome data assembly and quality.

Information	Value
Genome size (Mb)	34.75
DNA coding (bp)	18,279,031
DNA G+C (%)	53
DNA scaffolds	57
Coverage	60X
tRNA	30

Comparing *F. erecta* with related species of Chaetotryriales it was observed that all genomes used in this study including the reference species are similar in size. The genome size of *Fonsecaea* species varied between 33.39 and 35.23 Mb. The *F. monophora* genome is nearly 1.84 Mb larger in size following of the plant associate species *F. erecta* with 34.75, while the reference genome of the species plant associated *Cladophialophora yegresii* presented a reduced size of 27.9 Mb. The total number of initial predicted genes in *Fonsecaea* varied between 11,681 in *F. nubica* to 12,527 in *F. pedrosoi*; between 10,944 (93.69%) and 11,948 (96.59%) of the genes identified as conserved hypothetical proteins. In addition, repetitive element identification was considered to be low in *Fonsecaea* siblings, ranging from 1.06 to 1.13% in *F. multimorphosa* to 1.93% in *F. monophora* (Table 2).

TABLE 2: Genome studied

Species	Strains	Source	Geography	GeneBank genome	Genome size (Mb)	G+C content (%)	Repetitive elements	Hypothetical proteins
Capronia semiimmersa	CBS 27337	Human chromoblastomycosis	Brazil	JYCC00000000.1	31.62	53.7	0.71	92,23
Cladophialophora bantiana	CBS 173.52	Human chromoblastomycosis	USA	JYBT00000000.1	36.72	51.3	4.47	93,10
Cladophialophora carrionii	CBS 160.54	Human chromoblastomycosis	Australia	PRJNA185784	28.99	54.3	1.15	91,98
Cladophialophora immunda	CBS 834.96	Human skin lesion	USA	JYBZ00000000.1	43.03	52.8	2.24	93,00
Cladophialophora psammophila	CBS 110553	Gasolin-polluted soil	Netherlands	AMGX00000000.1	39.42	50.6	6.92	82,89
Cladophialophora yegresii	CBS 114405	*Stenocereus griseus* cactus	Venezuela	AMGW00000000.1	27.90	54	1.13	79,56
Coniosporium apollinis	CBS 100218	Pentelic marble	Greece	AJKL00000000.1	28.65	52.1	–	85,72
Fonsecaea erecta	CBS 125763	Living plant	Brazil	LVYI00000000.1	34.75	53	1.74	90,54
Fonsecaea monophora	CBS 269.37	Human chromoblastomycosis	South America	LVKK00000000.1	35.23	52.22	1.93	93,88
Fonsecaea multimorphosa	CBS 980.96	Cat brain abcess	Australia	LVCI00000000.1	33.39	52.64	1.06	96,34
Fonsecaea multimorphosa	CBS 102226	Decaying trunk palm tree	Brazil	PRJNA233317	33.45	52.6	1.13	96,59
Fonsecaea nubica	CBS 269.64	Human chromoblastomycosis	Cameroon	LVCJ00000000.1	33.79	52.46	1.59	93,69
Fonsecaea pedrosoi	ATCC 46428	Human chromoblastomycosis	South America	PRJNA233314	34.69	52.4	1.5	92,87
Fonsecaea pedrosoi	CBS 271.37	Human chromoblastomycosis	South America	PRJNA233314	34.69	52.4	1.5	92,87
Rhinocladiella mackenziei	CBS 650.93	Human cerebral phaeohyphomycosis	Saudi Arabia	JYBU00000000.1	32.47	50.4	3.49	92,48

Mitochondrial Genomes

Fonsecaea erecta CBS 125763^T and *F. pedrosoi* CBS 271.37^T mtDNA was assembled in one contig, measuring 25.7 and 25 Kb, respectively. The mtDNA of *F. monophora* assembled into eight contigs comprising 24.7 Kb, the mtDNA of *F. nubica* resulted in a single contig with 24.5 Kb and the mtDNA of *F. multimorphosa* CBS 980.96^T resulted in seven contigs with a size of 26.4 Kb. Although the gene composition of all mitochondrial genomes analyzed shared 16 protein coding genes involved in the respiratory chain and ATP synthesis, the synteny of these genes is not conserved, showing rearrangements when compared between *Fonsecaea* species and with the reference sequence of *E. dermatitidis* (Figure 2).

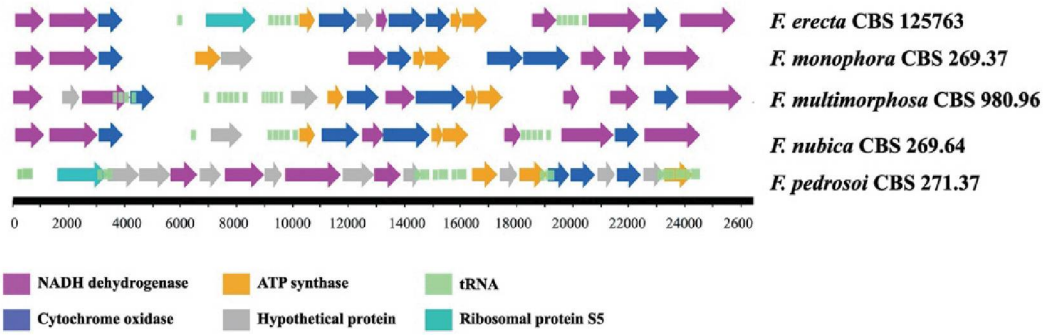


FIGURE 2: Mitochondrial genomes of *Fonsecaea* sibling species

Orthologous Gene Comparison among *Fonsecaea* Siblings

In order to study gene family evolution in *Fonsecaea*, we identified 20,713 orthologous gene clusters across 17 fungal genomes of environmental species and agents of subcutaneous and disseminated infection in animals and humans, including 9 species from the bantiana-clade and 2 from the carrionii- clade using *Coniosporium apollinis, Exophiala aquamarina,* and *Phialophora attae* as outgroup. The comparison showed a total of 5,727 of gene family clusters shared among *Fonsecaea* species, which was used to build a phylogenomic tree (Figure 3A).

The analysis showed strong support for published phylogenetic relationships between bantiana-and carrionii-clades in the order Chaetothyriales. Agents of chromoblastomycosis were distributed in the separate clades, with species associated with vertebrate infection clearly being distinct from environmental siblings. *Fonsecaea pedrosoi* and *F. nubica* causing chromoblastomycosis were closely affiliated with *F. monophora* involved in the same disease and as well as in brain infection. The plant-associated *F. erecta* is distinct from the clinical species. In addition, *F. multimorphosa*, an environmental species that once caused a feline cerebral abscess, formed a separate cluster with an environmental sibling (Figure 3A).

Cluster analysis was performed in the protein set of four *Fonsecaea* species: *F. erecta* CBS 125763^T, *F. multimorphosa* CBS 980.96^T (Leão et al., 2017), *F. monophora* CBS 269.37^T (Bombassaro et al., 2016) and *F. nubica* CBS 269.64^T (Costa et al., 2016). The core genome comprised almost 70% (~8,000) of the genes (Table S1); the number of accessory genes (Table S2) in *F. erecta* was less than 20%, with specific genes (Table S3) lower than 10% (~2,000) (Figure 3B).

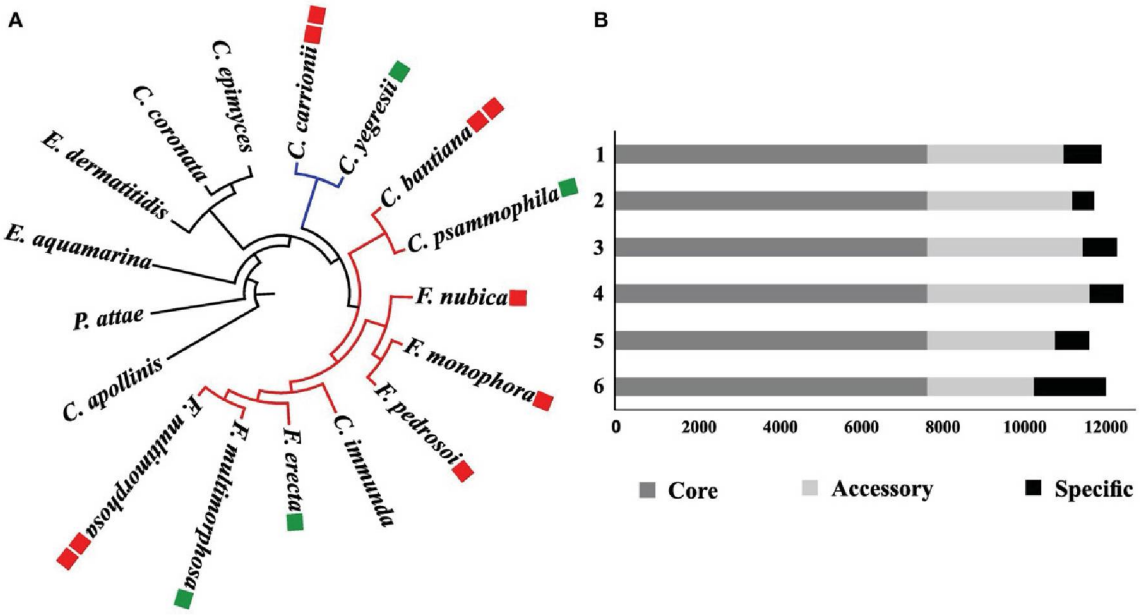


FIGURE 3: Phylogenomic analysis among *Fonsecaea* sibling species. (A) Phylogenetic tree based on the orthologous gene clusters. The bantiana-clade is indicated by a red line and the carrionii-clade by a blue line. Species names in green boxes are environmental strains, in red boxes agents of chromoblastomycosis and cerebral infection. (B) Gene cluster distribution in *Fonsecaea* siblings. Clusters of orthologous genes are showed in colors: dark gray core genes; light gray accessory genes, black specific genes. 1. *F. monophora* CBS 269.37, 2. *F. multimorphosa* CBS 980.96. 3. *F. multimorphosa* CBS 102226, 4. *F. pedrosoi* CBS 271.37, 5. *F. nubica* CBS 269.64, 6. *F. erecta* CBS 125763.

The gene core set was annotated with Eukaryotic Orthologous Group (Table S1). A total of 6,775 genes family's clusters were redundantly assigned into KOG classifications, of which 2,259 genes were annotated as poorly characterized proteins, 1,382 assignments were in the category of Information Storage and Processing, 2,128 in the Cellular Processes and Signaling category and 3,265 in the Metabolism category (Figure 4). Of the eight KOG sub-classifications allocated in the Metabolism category, 718 genes were annotated to the Secondary Metabolites Biosynthesis, Transport and Catabolism [Q], 566 genes annotated to Lipid Transport and Metabolism [I] and 552 genes annotated to Energy Production and Conversion [C], composing the three more relevant classifications in this category. Moreover, in the Cellular Process and Signaling category, 631 genes were annotated with 51% proteins distributed in the Post- translational Modification Protein Turnover Chaperones class [O] with 450 genes annotated and the class Signal Transduction Mechanisms [T] and 326 genes in the Intracellular Trafficking Secretion and Vesicular Transport [U], composing the top three classifications in this category to each species analyzed (Figures 5A,B).

In the Q class (Figure 4) significant domains were identified as being common among *Fonsecaea* clinical and plant-associated species. The catalytic domain related to zinc-containing alcohol dehydrogenase (ADH) (IPR002328, IPR013149, IPR013154) is the most abundant domain in the *Fonsecaea* siblings which has been implicated in many biochemical pathways with a role in pathogenicity, stress and intoxication and were previously reported to be expanded in black yeast species (Das and Vasudevan, 2007; Grahl et al., 2011; Strommer, 2011; Teixeira et al., 2017). In addition, flavin-containing monooxygenases (FMOs) (IPR000960) constitute a family of xenobiotic-metabolizing enzymes that are widely present in *Fonsecaea* species. Moreover, related to metabolic processes, some protein domains in Q class were related to the biosynthesis of melanin and were widely found among *Fonsecaea* siblings: IPR011141, IPR019587, and IPR004235 with a role in DHN-melanin pathway, IPR002227 related to DOPA-melanin pathway, and IPR005708, IPR005955, and IPR005956 related to L- tyrosine degradation pathway. Some key enzymes involved in melanin biosynthesis were observed, including the family multicopper oxidases (MCOs) (IPR011706, IPR011707, and IPR001117). This family includes laccases, ferroxidases, bilirubin oxidases and ascorbate oxidases (Hoegger et al., 2006). Likewise, the superfamily aldehyde dehydrogenase (ALDH) (IPR015590, IPR008274) represents a divergently related group of enzymes that metabolize a wide variety of endogenous and exogenous aldehydes (Vasiliou et al., 2000).

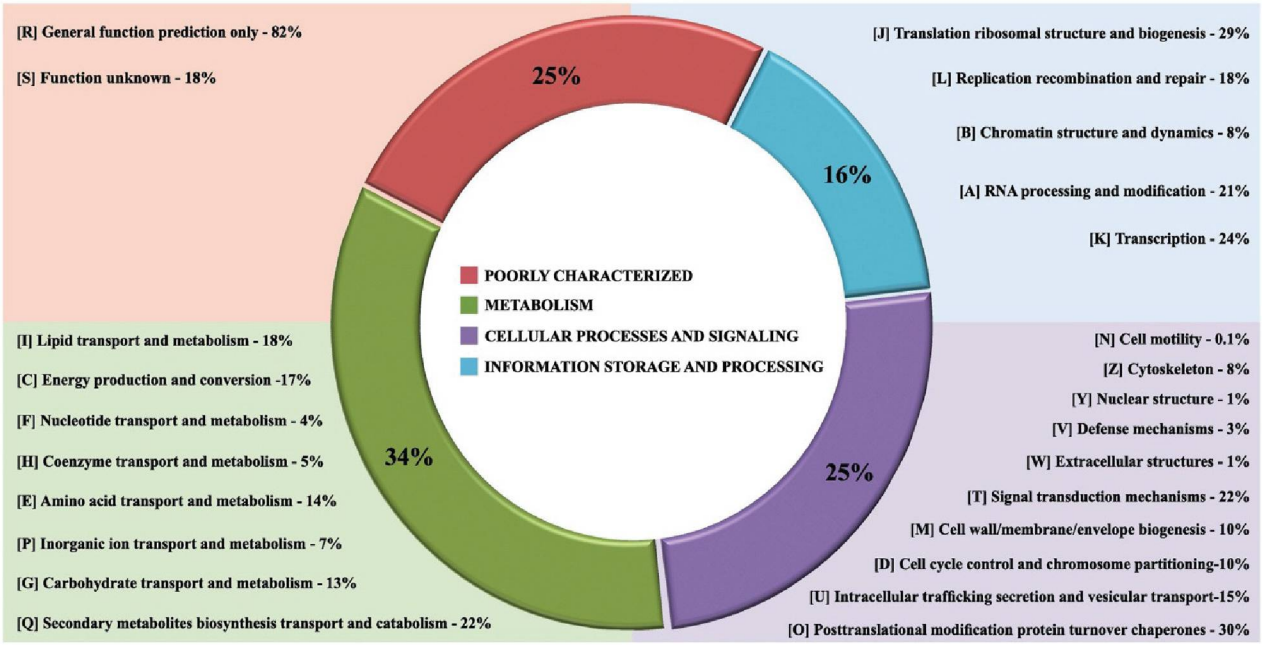


FIGURE 4: The core gene annotation from Eukaryotic Orthologous Group (KOG) in *Fonsecaea* species. KOG annotation by categories: In red poorly characterized proteins; in green metabolism category, in purple cellular processes and signaling category and processing and in blue category of information storage.

In the T class (Figure 4) a caspase-like domain (IPR029030) and peptidase C14A, caspase non-catalytic subunit p10 (IPR002138), which are broadly classified as cysteine peptidases, were identified among the *Fonsecaea* species studied. Cysteine- dependent aspartyl-specific protease is mainly involved in mediating cell death processes, while caspases also have roles other than in apoptosis, e.g., caspase-1 (interleukin-1 beta convertase), which is involved in inflammatory processes (Abraham and Shaham, 2004; Lamkanfi et al., 2007). In the T class, small GTPases family (IPR001806), an independent superfamily within the larger class of regulatory GTP hydrolases (Bourne et al., 1990), was observed in *Fonsecaea* siblings ARF type (IPR02456), SAR1 type (IPR006687), ARF/SAR1 type (IPR006689) and Small GTPase, Ras type (IPR020849). Within class O, post-translational modification protein turnover chaperones are involved in folding, maintenance, intracellular transport and degradation of proteins as well as in facilitating cell signaling. Many heat shock protein (Hsp) families have been identified in this study, such as Hsp20-like chaperone (IPR008979), Hsp40/DnaJ peptide-binding (IPR008971), Heat shock protein Hsp90, N-terminal (IPR020575), Heat shock protein Hsp90 family (IPR001404), Heat shock protein Hsp90, conserved site (IPR019805). Likewise, using the functional annotation based on Gene Ontology (GO), a total of 7,392 genes were assigned (Figure S1). Of these genes, 409 were redundantly assigned into Cellular Component Ontology, 4,752 into Molecular Function Ontology and 2,231 into Biological Process Ontology. Most of the genes were annotated to binding (434) and oxyreductase (277) and methyltransferase (187) activity in the Molecular Function Ontology. In the Biological Process the overrepresented functions were metabolic processes (495), transport (239), and biosynthetic processes (195). The less represented ontology classes were membrane (87), protein complex (69) and chromosome (32) in Cellular Component Ontology.

Judging from these analyses, the presence and abundance of these functional domains could be related to the ecology of this agents, i.e., Siderophore iron transporter (GO:0015892, IPR010573) recovered by cells either by the reductive system or by specific transporters able to internalize the siderophore- iron complex (Itoh et al., 2000) and the Quinoprotein amine dehydrogenase (IPR011044), which is related to extremotolerance, this domain has been shown to interact with series of metal ions in anhydrous organic media (Matthews and Sunde, 2002).

In addition, GO enrichment analyses were used to determine the functional characteristics in the studied species (Table S4). It was observed that *F. erecta* presents a mechanism to regulate the use of nitrogen (GO: 0006808), a feature that may favor its survival in plants. Likewise, *F. erecta* and *C. yegresii* shared GO enrichments related to degradation of phenolic compounds such as L-phenylalanine catabolic process (GO: 0006559), tyrosine catabolic process (GO: 0006572) and homogentisate 1,2-dioxygenase activity (GO: 0004411). On the other hand, *C. carrionii* and *F. pedrosoi* exhibited an overrepresentation of the following GO terms: cellular response to iron ion starvation (GO: 0010106), siderophore biosynthetic process (GO: 0019290) and ferric triacetylfusarinine C transport (GO: 0015686) that are involved in iron metabolism.

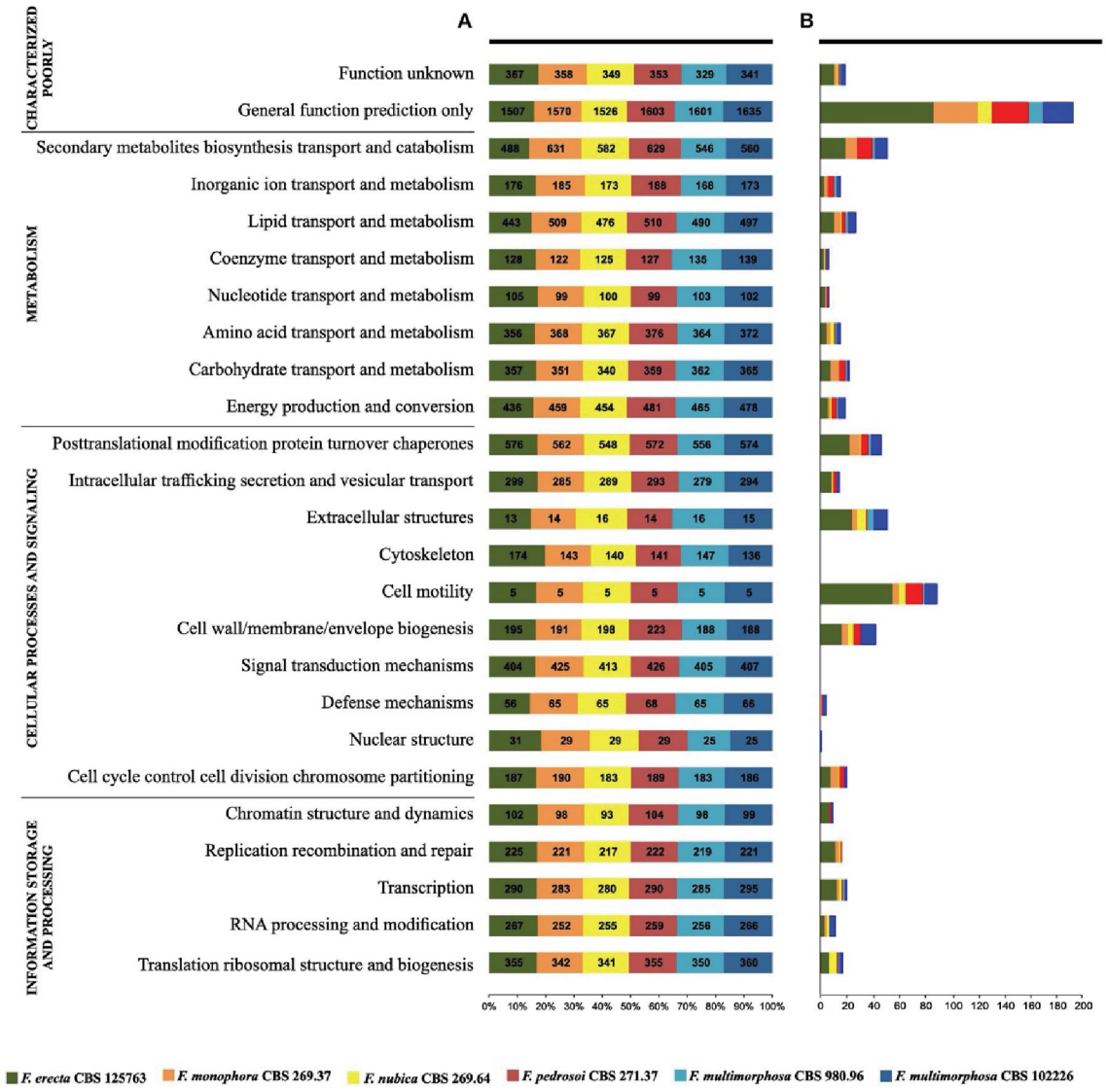


FIGURE 5: Gene families shared between *Fonsecaea* siblings based on annotation from Eukaryotic Orthologous Group (KOG). (A) The siblings are summarized in colored boxes. (B) Specific gene annotation among *Fonsecaea* siblings.

Species-Specific Genes

The gene families deduced by KOG annotations for each *Fonsecaea* species studied were presented in the Figure 5A. Considering the specific genes observed in each species (Figure 5B), the plant-associated *F. erecta* presented the largest number of genes related to General function prediction and Secondary metabolites biosynthesis transport and catabolism. The species had a Leucine-rich repeat variant (IPR004830, PF13855) and Universal stress protein signature (USP) (PF00582) domains. Moreover, the family fungal Fucose- specific lectin (IPR012475) and the domain Jacalin-type lectin domain profile (IPR001229) involved in metabolism of lectins also were found in this specie (Table S3). Zinc finger domains (Znf) are shared by *Fonsecaea* sibling, but some domains are present in specific proteins to some species (Table S3). The Znf are relatively small protein motifs, which contain multiple finger-like protrusions that make tandem contacts with their target molecules. There are many superfamilies of Znf motifs, varying in both sequence and structure (Matthews and Sunde, 2002).

Virulence-Related Genes

Using PHI (Pathogen Host Interactions data base) (Winnenburg et al., 2007), genes of species belonging to the bantiana- and carrionii-clades were classified into categories considering virulence and pathogenicity. In total 3,865 genes were divided into 3 categories: Lethal 2,801 genes, Increased virulence (hypervirulence) 1,013 genes and Effector (plant avirulence determinant) 51 genes (Table S5). According to this analysis, 3,614 genes were

present in all species studied that includes different transcription factors. On the other hand, 251 genes were limited to one or more species such as *Xeg1* observed in *F. erecta* and *Gr-VAP* gene in *C. yegresii* and *F. erecta.* In the class hypervirulence, all the analyzed species share the transcription factor *Amr1*, a gene described in *Alternaria brassicicola* this is an obligate plant pathogen as an inducer of melanin biosynthesis and associated with pathogenesis, suggesting that it is an important gene for the species to be a competitive saprophytic and also as a parasite (Cho et al., 2012). Moreover, the *RpfF* gene involved in pathogenesis of opportunistic pathogens (Suppiger et al., 2016) was observed in *F. multimorphosa*, *F. pedrosoi*, *F. monophora,* and *F. nubica* from the bantiana-clade and it was absent in the species from carrionii-clade (Table 3).

TABLE 3: *Fonsecaea* and *Cladophialophora* specific genes annotated in the PHI base.

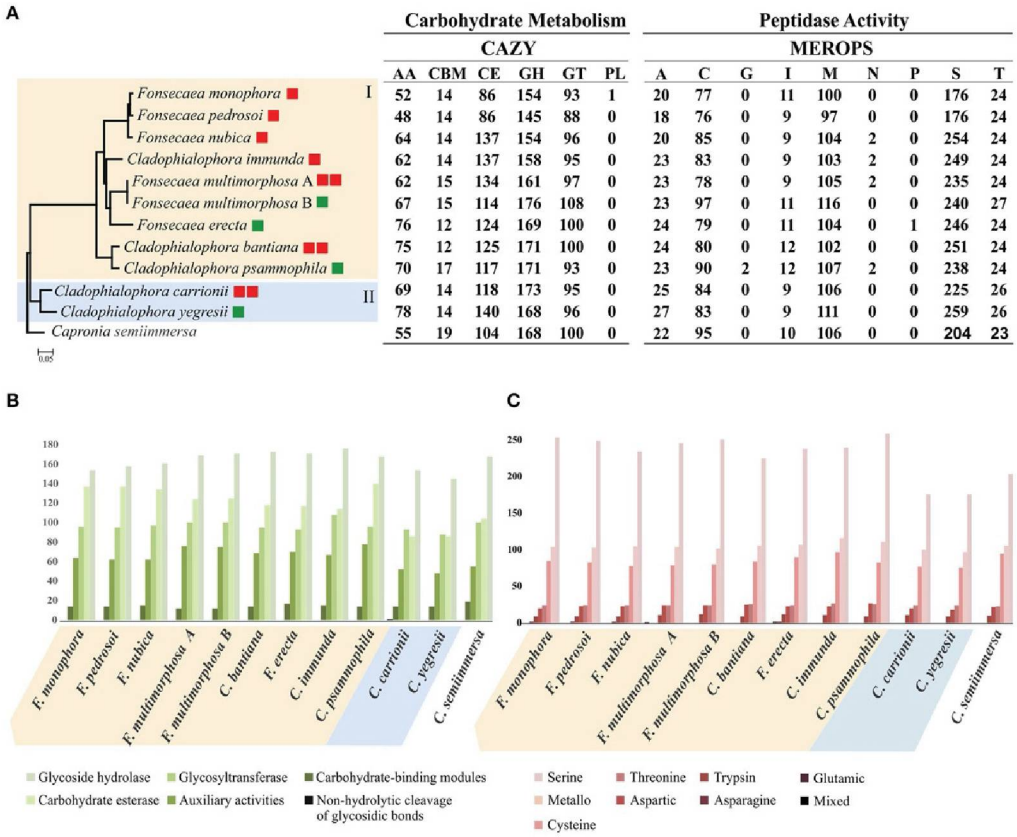
Species	Genes	Accession	Function in pathogenicity
F. erecta	*Xeg1*	OAP56881.1	Triggered defense responses including cell death Ma et al., 2015
F. erecta	*GzHOMEL040*	OAP56314.1	Transcription factor Son et al., 2011
F. erecta	*GzZC062*	OAP64706.1	Transcription factor Son et al., 2011
F. nubica	*GzC2H089*	OAL38924.1	Transcription factor Son et al., 2011
F. pedrosoi	*RSc1356*	XP013280599.1	Gene effector in plant infection Pensec et al., 2015
F. monophora	*RSc1356*	OAG34226.1	
F. nubica	*RSc1356*	OAL31073.1	
*F. multimorphosa**	*TcGALE*	OAL25194.1	Responsible for conidiation and mycelial development El-Ganiny et al., 2010
*F. multimorphosa***	*COS1*	XP_016633043.1	Function as a transcriptional regulator controlling genes responsible for conidiation Zhou et al., 2009
F. erecta	*Gr-Vap1*	OAP625883.1	Defense-related programmed cell death in plant cells Lozano-Torres et al., 2012
C. yegresii	*Gr-Vap1*	XP_007761267.1	
F. monophora	*RpFf*	OAG36832.1	Regulation of biofilm formation, colony morphology, proteolytic activity, and virulence Suppiger et al., 2016
F. pedrosoi	*RpFf*	XP_013286584.1	
F. nubica	*RpFf*	OAL34548.1	
*F. multimorphosa**	*RpFf*	OAL19303.1	
*F. multimorphosa***	*RpFf*	XP_016628078.1	
C. yegresii	*AMR1*	XP_007752625.1	Inducer of melanin biosynthesis Cho et al., 2012
C. carrionii	*AMR1*	KIW73572.1	
F. monophora	*AMR1*	OAG33974.1	
F. pedrosoi	*AMR1*	XP_013282497.1	
F. nubica	*AMR1*	OAL32231.1	
*F. multimorphosa**	*AMR1*	OAL18751.1	
*F. multimorphosa***	*AMR1*	XP_016627577.1	
F. erecta	*AMR1*	OAP58408.1	
C. immunda	*AMR1*	XP_016244825.1	
C. bantiana	*AMR1*	XP_016622751.1	
C. psammophila	*AMR1*	XP_007742386.1	

**CBS 980.69 isolated form brain disseminated infection in cat.*
***CBS 102226 environmental isolate.*

Fungal Lifestyles Expressed in Peptidases and Carbohydrate-Active Enzymes

The bantiana-and carrionii-clades were annotated with carbohydrate-active enzymes (CAZymes) database resulting in 5,058 genes encoding putative CAZymes, comprising 723 auxiliary activities (AA), 155 carbohydrate binding module (CBM), 1,318 carbohydrate esterases (CE), 1,800 glycoside hydrolases (GH), 1,061 glycosyl transferases (GT) and 1 polysaccharide lyase (Figure 6). The phylogenomic tree showing the carbohydrate and peptidase metabolism content in bantiana-and carrionii-clades was presented in the Figure 6A. In both clades CAZymes associated with degradation of polysaccharides such as chitin, hemicellulose, glucans and pectin were the largest enzyme families. The number of glycoside hydrolase 43 (GH43) enzyme family related to pectin and hemicellulose degradation was higher in *F. erecta* than the other species (Table S6). In addition, in the bantiana-clade the activity carbohydrate esterase (CE) was higher than in the carrionii-clade, while no polysaccharide lyase (PL) was identified in *Fonsecaea* sibling genomes, only one in *C. carrionii* (Figure 6B).

Peptidase-encoding genes were predicted using the MEROPS database (Rawlings et al., 2015) (Table S7). The bantiana- and carrionii-clades were predicted to produce a wide repertoire of different endo- and exopeptidases. We identified for both clades 5,266 peptidases, of which the largest families were serine peptidases (2,549), metallo peptidases (1,155) and cysteine peptidases (912), wherein peptidase family S1 containing Serine endopeptidase was higher in the bantiana-clade (Figure 6C). The relation between CAZY and MEROPS) in bantiana- and carrionii-clades is shown in Figure 6 (family-specific classification shown in Tables S6, S7). The species in bantiana- and carrionii-clades showed similarity in gene contents, sharing different carbohydrate metabolism and peptidase genes ($p = 0.00398$; Mann-Whitney U test), suggesting that these fungi are able to degrade plant and animal substrates.



	AA	CBM	CE	GH	GT	PL	A	C	G	I	M	N	P	S	T
Fonsecaea monophora	52	14	86	154	93	1	20	77	0	11	100	0	0	176	24
Fonsecaea pedrosoi	48	14	86	145	88	0	18	76	0	9	97	0	0	176	24
Fonsecaea nubica	64	14	137	154	96	0	20	85	0	9	104	2	0	254	24
Cladophialophora immunda	62	14	137	158	95	0	23	83	0	9	103	2	0	249	24
Fonsecaea multimorphosa A	62	15	134	161	97	0	23	78	0	9	105	2	0	235	24
Fonsecaea multimorphosa B	67	15	114	176	108	0	23	97	0	11	116	0	0	240	27
Fonsecaea erecta	76	12	124	169	100	0	24	79	0	11	104	0	1	246	24
Cladophialophora bantiana	75	12	125	171	100	0	24	80	0	12	102	0	0	251	24
Cladophialophora psammophila	70	17	117	171	93	0	23	90	2	12	107	2	0	238	24
Cladophialophora carrionii	69	14	118	173	95	0	25	84	0	9	106	0	0	225	26
Cladophialophora yegresii	78	14	140	168	96	0	27	83	0	9	111	0	0	259	26
Capronia semiimmersa	55	19	104	168	100	0	22	95	0	10	106	0	0	204	23

FIGURE 6: Analysis of carbohydrate and peptidase metabolism content in bantiana-and carrionii-clades. (A) Phylogenomic tree of bantiana- (I) and carrionii-clades (II) *Fonsecaea multimorphosa* (A) CBS 980.69. *Fonsecaea multimorphosa.* (B) CBS 102.226. All nodes on the phylogeny were supported by bootstrap values of 100% and the letters indicate ancestors. Species names followed by boxes: in green environmental strains, in red agents of chromoblastomycosis and cerebral infection. The ratio of MEROPS enzymes to CAZY enzymes for each genome is shown in the last column. (B) CAZY annotation: categories include AA (auxiliary activities), CBM (carbohydrate-binding modules), CE (carbohydrate esterase), GH (glycoside hydrolase), GT (glycosyltransferase) and PL (non-hydrolytic cleavage of glycosidic bonds). (C) MEROPS annotation: categories include A (aspartic), C (cysteine), G (glutamic), I (trypsin), M (metallo), N (asparagine), P (mixed), S (serine), and T (threonine).

Protein Family Expansion and Contraction

Protein domain expansions and contractions were inferred from the abundance of protein domains predicted by InterProScan searches and statistically tested by CAFE v3.0 (De Bie et al., 2006) conserved site (IPR002328)

showed an expansion in the ancestor of *F. pedrosoi*, *F. monophora* and *F. nubica*. Similarly, expansions in *F. erecta* and *C. bantiana* were observed in the protein family peptidase M20 (IPR002933 and IPR011650) and in the CHAT domain (IPR024983) related to caspases (Figure 7, Table S8).

Moreover, two domains associated with the glyoxal pathway, the glyoxalase I (IPR018146) and the glyoxalase/fosfomycin resistance/dioxygenase (IPR004360) are expanded in the ancestor of *F. pedrosoi, F. monophora*, and *F. nubica*. This enzyme catalyzes the first step of the glyoxal pathway, and in addition to its role in detoxifying glyoxal it may have other roles in stress response (Kim et al., 1993; Yim et al., 2001). Further this ancestor showed expansion in the aminoglycoside phosphotransferase (IPR002575) domain (Figure 7, Table S8); this domain consists of bacterial antibiotic resistance proteins, which confers resistance to various aminoglycosides (Trower and Clark, 1990; Chow, 2000).

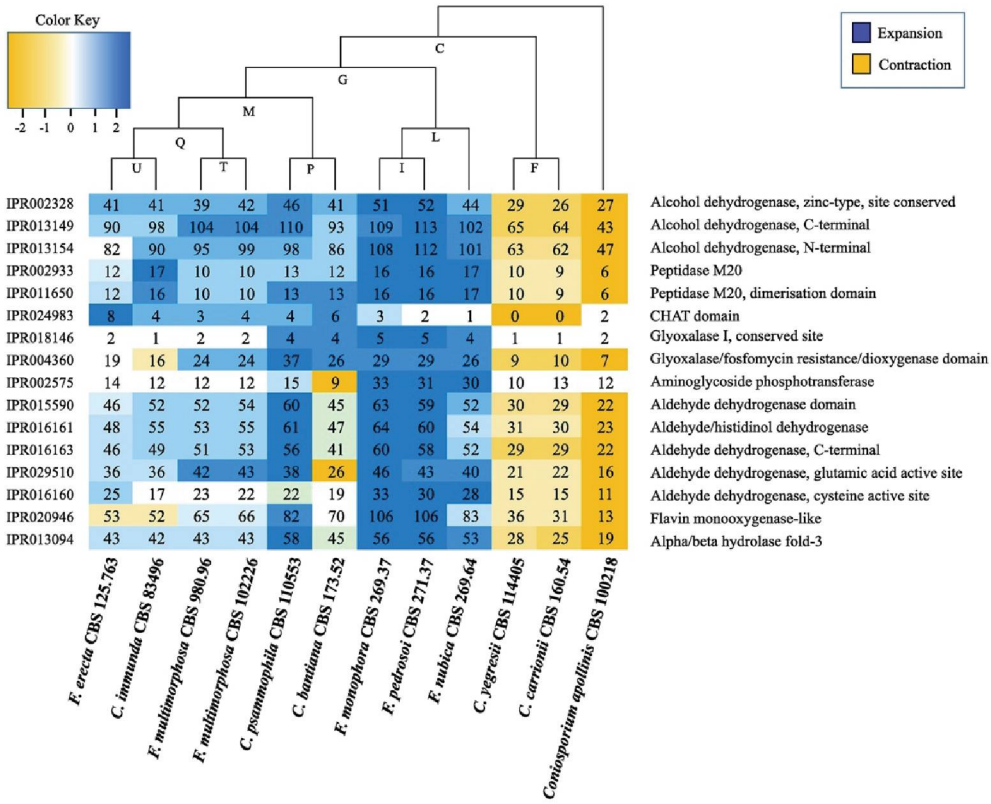


FIGURE 7: Interpro domains in *Fonsecaea* siblings. The letters indicate values for ancestry. The species are organized in phylogenetic order.

Virulence of *Fonsecaea* Siblings

Survival tests using *Galleria mellonella* larvae as a model infected with *F. pedrosoi* ATCC 46428 and CBS 271.37T, *F. erecta* CBS 125763T, and *F. monophora* CBS 102248 showed a low larvae survival rate when compared to control groups. Larvae infected with *F. erecta* had a lower survival rate (**Figure 8A**). The infected larvae from the tested groups developed varying degrees of melanization, whereas the controls (PBS and SHAM) (Thomaz et al., 2013) did not present any melanization (**Figure 8B1–6**). Melanization could be observed from the first day post- inoculation in all tested groups. Histopathology (**Figure 8C**) showed the presence of pigmented nodules and granulomata in the tissue of infected larvae. Based on these results it was concluded that *F. erecta* CBS 125763T is potentially able to infect animal hosts. In order to find out how *F. pedrosoi* and *F. erecta* conidia and hyphae trigger macrophage activation, microbicidal activity and TNF-α production was measured. *Fonsecaea erecta* hyphae are more resistant to macrophage destruction than *F. pedrosoi* after 24h of co-culturing with macrophages in vitro (**Figure 9**). No difference was observed when macrophages were co-cultured with conidia from both *Fonsecaea* species (**Figure 9A**). Only hyphae from both *Fonsecaea* species were able to induce TNF-α secretion (**Figure 9B**). In vivo assay using BALB/c mice as a model infected by pathogenic species *F. pedrosoi* and plant associated species F. erecta showed ulcerative and plaque type lesions. However, the *F. pedrosoi* infected mice showed lesions with dark plaque while F. erecta infected mice presented higher areas of edema (**Figure 10A**). All groups showed high levels of fungal burden at 7 dpi followed by reduction of viable fungi in the injured area (**Figure 10B**). The levels of cytokines were similar in both groups, except to IL-1β levels in *F. erecta* infected mice that increase lately when compared with *F. pedrosoi* infected mice (**Figures 10C–F**).

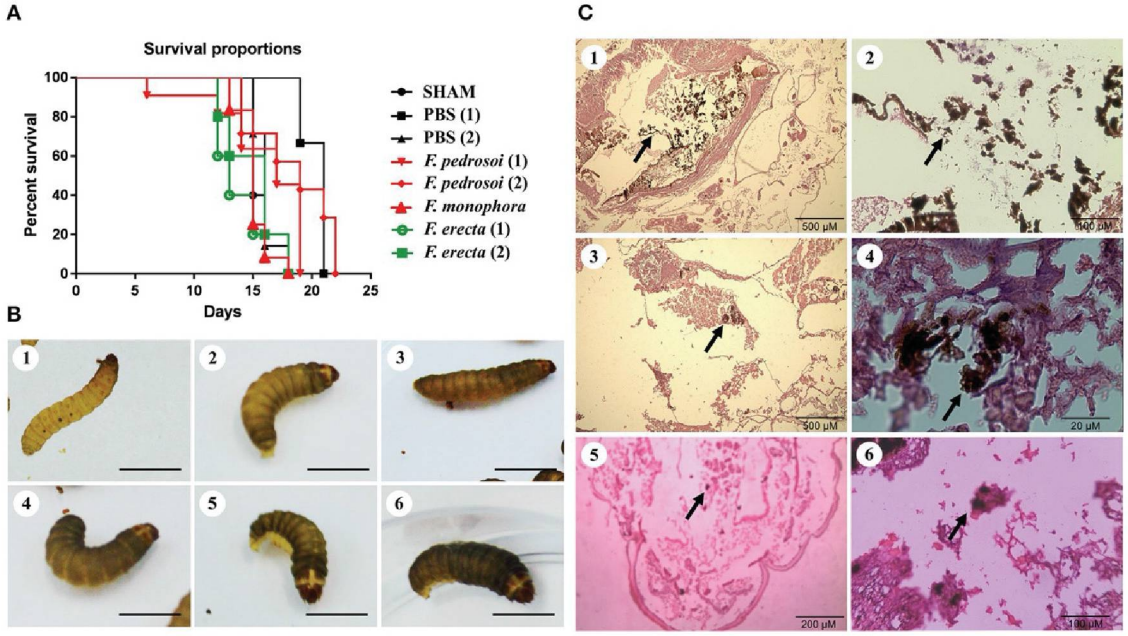


FIGURE 8: Virulence of *Fonsecaea* siblings using *Galleria mellonella* larvae. (A) Survival of *G. mellonella* larvae infected with *Fonsecaea* species. (B) Melanization of *G. mellonella* larvae infected with *Fonsecaea* species: (1, 2). Controls: SHAM and PBS; (3). *Fonsecaea erecta* CBS 125763; (4). *Fonsecaea monophora;* (5). *Fonsecaea pedrosoi* CBS 271.37; (6). *Fonsecaea pedrosoi* ATCC 46428. (C) Histology of infected tissue of *G. mellonella* with *Fonsecaea* species. The internal structures were fixed, embedded in paraffin and stained with PAS. Black arrows show hyphae spreading through the larva tissue. (1, 2). *Fonsecaea erecta* CBS 125763; (3, 4). *Fonsecaea pedrosoi* CBS 271.37; (5, 6). *Fonsecaea monophora* CBS 102248.

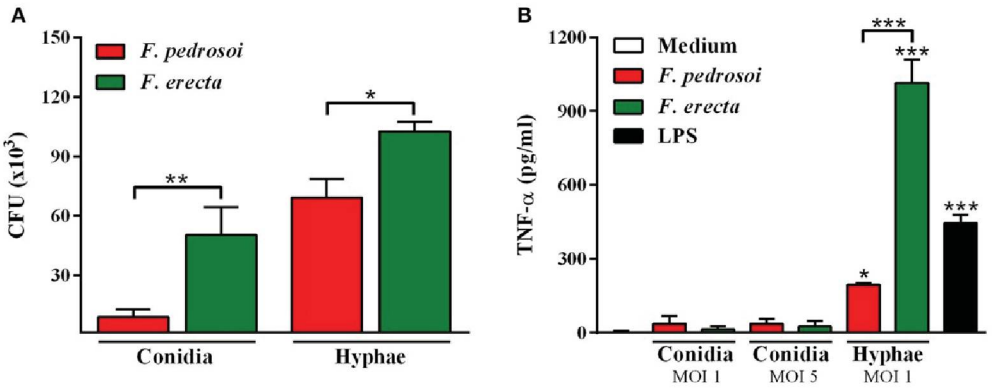


FIGURE 9: Fungal burden and production of pro-inflammatory cytokines of *F. pedrosoi* and *F. erecta.* (A) J774 murine macrophages (J774) were co-cultured with conidia or hyphal fragments, in the MOI 1 during 24 h. CFU data showed faster clearance of inoculated conidia from *F. pedrosoi* than *F. erecta.* (B) High levels of TNF-α were observed in macrophage co-culture with hypha, but not with conidia. Data were analyzed by one-way ANOVA with Tukey's post-hoc test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with F. pedrosoi; or compared with non-infected macrophages.

Potential Virulence Genes Related to Human Infection

An analysis of common genes related to the casual agents of disease showed 43 gene clusters shared by agents of systemic infection and 32 gene clusters related to (sub)cutaneous infection. Annotation of these clusters in *Fonsecaea* revealed 33 domains related to disseminated infection and 24 domains related to subcutaneous infection (Table 4). In the agents of systemic infection a high frequency was noted of the domains DJ-1/PfpI family (IPR002818) and glyoxalase/fosfomycin resistance protein/dioxygenase superfamily (IPR004360) related to glyoxal pathway, which have important roles in detoxifying glyoxals (Lee et al., 2013) and domains related to flavin proteins, such as monooxygenase- like flavin-binding (IPR020946) and flavin-containing amine oxidoreductase (IPR002937), and the family multicopper oxidases (MCOs) (IPR011706, IPR011707, and IPR001117) that includes laccases, ferroxidases, bilirubin oxidases and ascorbate oxidases (Hoegger et al., 2006). The subcutaneous agents shared a

number of domains related to enzymes and transporters (Table 4), such as major facilitator, sugar transporter-like (IPR005828) and major facilitator superfamily IPR011701), aggregating several families of transporters. Among them a siderophore transporter, RhtX/FptX family and the yellow-like family is a gene class characterized by the presence of a major royal jelly protein (MRJP) domain (Ferguson et al., 2011) with multiple physiological and development functions in insects (Drapeau et al., 2003), such as synthesis of melanin pigments and sex-specific reproductive maturation. A large variety of enzymes such as acyltransferases (IPR002656), hydrolases (IPR013094 and IPR000757), proteases, lipases, permeases (IPR004841) and dehydrogenases (IPR002347) were observed. Nevertheless, also domains related to invasion of the plant tissue were observed, such as cutinase (IPR000675) (Dickman et al., 1989) and LysM domain related to chitinase (IPR018392) (Gruber and Seidl-Seiboth, 2012).

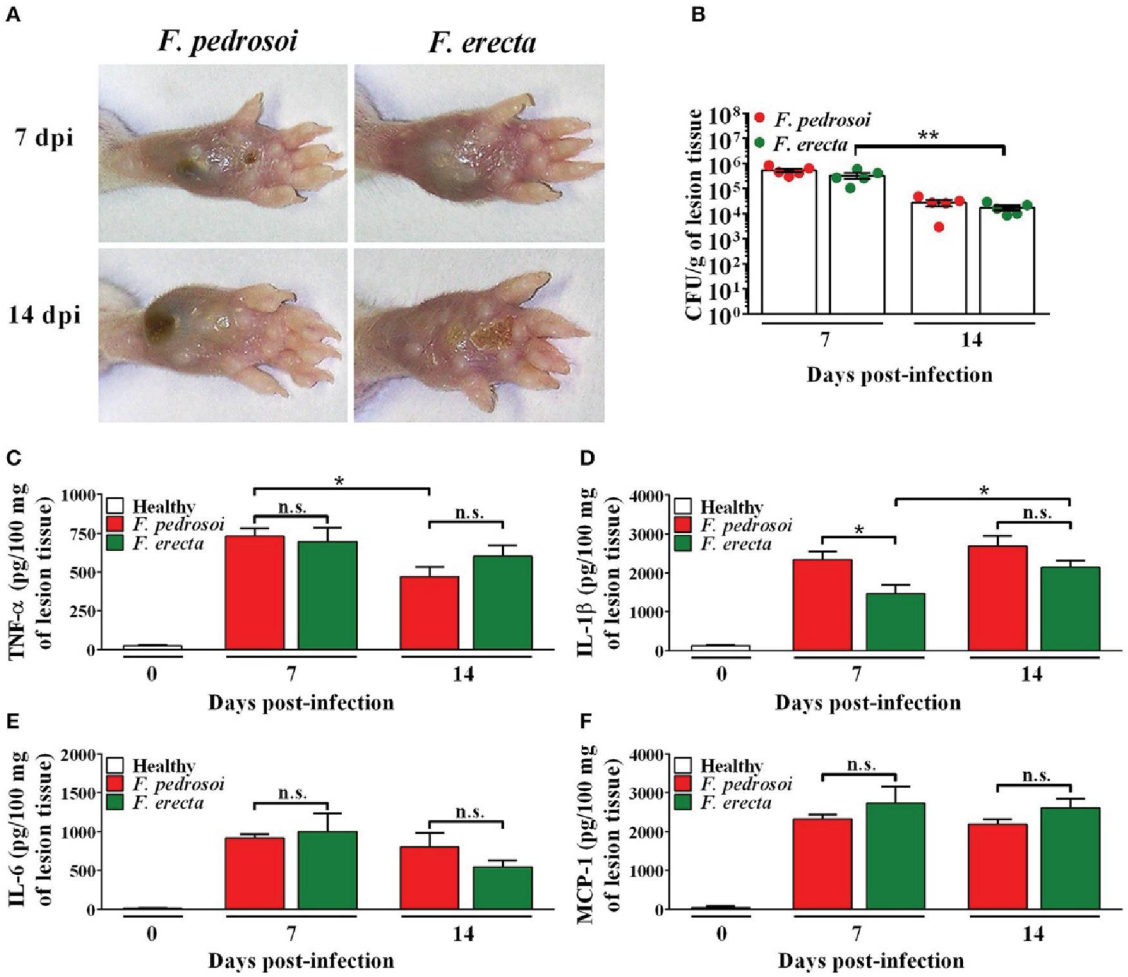


FIGURE 10: Virulence and immunostimulatory potential test of *Fonsecaea* sibling species using BALB/c mice as a model. (A) Macroscopic aspect of the disease. (B) CFU data showed a high tissue fungal burden of F. erecta which decline over the course of the infection. (C–F) At 7 and 14 days post-infection, high levels of TNF-α, IL-1β, IL-6 and MCP-1 were observed similarly in the footpad of mice infected with F. pedrosoi or F. erecta. Data were analyzed by two-way ANOVA with Tukey's post-hoc test. *$p < 0.05$ and **$p < 0.01$ between groups indicated by brackets; n.s.: not significantly.

TABLE 4: Prediction of virulence domains related to systemic and (sub)cutaneous infection.

Systemic infection domains (Domain/ID access)	Function	(Sub)cutaneous infection (Domain/ID access)	Function
AhpC/TSA Family/IPR000866	Antioxidant activity	Acyltransferase family/IPR002656	Transferase activity
Alpha/beta hydrolase fold/IPR000866	Catalytic activity	Alpha/beta hydrolase fold/IPR013094	Hydrolase activity
Amidase/IPR023631	Hydrolase activity	Amino acid permease/IPR004841	Transmembrane transport
Amidohydrolase Family/IPR006680	Hydrolase activity	Asp/Glu/Hydantoin racemase/IPR015942	Nitrogen compound metabolic process
C-terminal of 1-Cys peroxiredoxin/IPR019479	Peroxiredoxin activity	Cutinase/IPR000675	Hydrolase activity
DJ-1/PfpI Family/IPR002818	Glyoxalase	Flavin-binding monooxygenase-like/IPR020946	NADP binding
Unknown function (DUF1772)/IPR013901	Anthrone oxygenases	Fungal specific transcription factor/IPR007219	Zinc ion binding
Eukaryotic aspartyl protease/IPR033121	Proteolytic enzymes	Fungal Zn(2)-Cys(6) binuclear cluster/IPR001138	Zinc ion binding
FAD binding domain/IPR003953	Catalytic activity	Glutathione-dependent formaldehyde activating enzyme/IPR006913	Carbon-sulfur lyase activity
Flavin-binding monooxygenase-like/IPR020946	Metabolize xenobiotics	Glycosyl hydrolases family 16/IPR000757	Hydrolase activity
Flavin amine oxidoreductase/IPR002937	Oxidoreductase activity	LysM domain/IPR018392	Binding chitin
Fumarylacetoacetate (FAA) hydrolase Family/IPR011234	Catalytic activity	Major Facilitator Superfamily/IPR011701	Transmembrane transport
Fungal N-terminal of STAND proteins/IPR031348	Function is not known	Major intrinsic protein/IPR000425	Transporter activity
Fungal specific transcription factor/IPR007219	Zinc ion binding	Major royal jelly protein/IPR017996	Function is not known
Fungal specific transcription factor/IPR021858	Transcription factor	NAD dependent epimerase/dehydratase family/IPR001509	Catalytic activity
Fungal Zn(2)-Cys(6) binuclear cluster/IPR001138	Zinc ion binding	NADP oxidoreductase coenzyme F420-dependent/IPR028939	Catalytic activity
Glyoxalase/fosfomycin resistance protein/Dioxygenase superfamily/IPR004360	Catalytic activity	OTT_1508-like deaminase/IPR027796	Chromatin function
GMC oxidoreductase/IPR000172	Oxidoreductase activity	Oxidoreductase family, NAD-binding Rossmann fold/IPR000683	Oxidoreductase activity
GMC oxidoreductase/IPR007867	Oxidoreductase activity	Phosphotransferase enzyme family/IPR002575	Antibiotic resistance
Heterokaryon incompatibility protein (HET)/IPR010730	Preserve genetic individuality	Putative oxidoreductase C terminal/IPR013944	Oxidoreductase activity
Major Facilitator Superfamily/IPR011701	Transmembrane transport	Putative threonine/serine exporter/IPR010619	Catalytic activity
Multicopper oxidase/IPR001117	Oxidation-reduction	Pyrroline-5-carboxylate reductase dimerization/IPR029036	Dimerization domain
Multicopper oxidase/IPR011707	Copper ion binding	Sugar (and other) transporter/IPR005828	Transmembrane transporter
Multicopper oxidase/IPR011706	Oxidoreductase activity	Short chain dehydrogenase/IPR002347	Catalytic activity
NAD(P)H-binding/IPR016040	Catalytic activity	Threonine/Serine exporter, ThrE/IPR024528	Transmembrane transporter
NMT1-like Family/IPR011852	Protein receptors	—	—
Phenazine biosynthesis-like protein/IPR003719	Catalytic activity	—	—
Prion-inhibition and propagation IPR029498	Prion-inhibitory and propagation effect	—	—
Unknown function (DUF4243)/IPR025337	Function is not known	—	—
Sugar (and other) transporter/IPR005828	Transmembrane transporter activity	—	—
X-Pro dipeptidyl-peptidase C-terminal non-catalytic/IPR013736	Dipeptidyl-peptidase activity	—	—
X-Pro dipeptidyl-peptidase (S15 family)/IPR000383	Hydrolase activity	—	—

DISCUSSION

The genus *Fonsecaea* is located in the Chaetothyriales, family Herpotrichiellaceae and contains species of environmental saprobes on plants or plant debris and pathogenic species, associated with subcutaneous and deep infections in human and animal hosts. The environmental species are consistently distinct from the *Fonsecaea* spp. commonly founded as agents of disease. The same phenomenon of environmental next to pathogenic species is known in *Cladophialophora* (Vicente et al., 2014).

Phylogenomic analysis shows that *Fonsecaea* is intermingled with some *Cladophialophora* species, while *Cladophialophora* agents of chromoblastomycosis are located in a separate carrionii-clade (Figure 3A). The invasive potential of black yeast-like fungi is known to differ significantly between species (Badali et al., 2008; Seyedmousavi et al., 2011). Both clades with agents of chromoblastomycosis also contain non-pathogenic representatives. Formation of muriform cells in host tissue is considered as the hallmark of the disease, but some environmental species are able to produce such cells *in vitro* (Badali et al., 2008). Genomes of species of *Fonsecaea* under study are quite similar. An abundance of domains related to zinc-containing alcohol dehydrogenase (ADH) was observed (Figure 4), which have multiple biological functions such as detoxification.

Similar results have been reported for other black yeasts (Teixeira et al., 2017), suggesting that tolerance of extreme and toxic environmental habitats is a mainstay in the ecology of black yeast-like fungi. Likewise, the superfamily ALDH represents enzymes that metabolize a wide variety of endogenous and exogenous aldehydes (Lindahl, 1992; Vasiliou et al., 2000). The expansion of ALDH in the ancestor of *Fonsecaea* (Figure 7) indicates metabolic plasticity explaining a dual ecological ability, surviving hostile environments as well as mammal host tissue. Also Flavin-containing monooxygenases (FMOs) play a role in a wide variety of processes such as the detoxification of drugs, biodegradation of aromatic compounds, siderophore biosynthesis and biosynthesis of antibiotics (van Berkel et al., 2006), which could thus be further key executors in processes related to extremotolerance.

Gene Ontology (GO) annotation indicated presence of siderophore iron transporters in the core genome of *Fonsecaea* (Figure S1). Most fungi are able to produce and utilize intracellular siderophores as an iron storage compound (Eisendle et al., 2006). In *Candida albicans* siderophore transporter- defective mutants were clearly compromised in invading keratinocyte layers suggesting that siderophore uptake is required to epithelial invasion and penetration (Leon-Sicairos et al., 2015). The clinical species *C. carrionii* and *F. pedrosoi* show gene enrichment in cellular response to siderophore biosynthetic process and ferric triacetylfusarinine C transport which might play a role in virulence of these agents, as these genes were not enriched in the environmental species (Table S4). *Fonsecaea* siblings produce melanin via different pathways demonstrating that in these fungi the pathways are conserved (Chen et al., 2014; Teixeira et al., 2017). Melanin and carotenoids deposited in the cell walls are considered a putative virulence factor in established human pathogens such as *Histoplasma capsulatum*, *Sporothrix schenckii*, and *Cryptococcus neoformans*. Melanin is associated to the survival and competitive abilities of fungi in hostile environments (Nosanchuk et al., 2002; Mednick et al., 2005; Nosanchuk, 2005; Taborda et al., 2008) and also enhances tolerance of oxygenic burst in macrophages. Key enzymes involved in melanin biosynthetic pathways belong to the family Multicopper oxidases (MCOs) including laccases, ferroxidases, bilirubin, oxidases and ascorbate oxidases that catalyze the oxidation of a variety of substrates and mainly aromatic compounds (Hoegger et al., 2006). Laccases form the largest subgroup and are considered key enzymes of the DOPA melanin pathway (Walton et al., 2005). They are abundant in fungal genomes, related to their divergent physiological roles and differential regulation upon changing environmental conditions (Cañero and Roncero, 2008; Giardina et al., 2010). Teixeira et al. (2010) suggested that melanin pigments protect the fungus from the mammalian host's innate immune responses providing resistance to oxidizing agents and fungal cell death during phagocytosis. Melanin is important because has a role in the protection against antifungal drugs (van den Sande et al., 2007) and is significant in differentiation of the muriform cell, the invasive form of chromoblastomycosis.

The glyoxalase system consists of two consecutive enzymatic reactions (Glyoxalase I and II) with the terminal product D-lactate by metabolism of the physiological substrate methylglyoxal. The widespread distribution of glyoxalase in prokaryotic and eukaryotic cells suggests it fulfills a function of fundamental importance to life. Inhibition of the glyoxalase system leads to methylglyoxal accumulation to toxic levels. It has been implicated in control of cell growth and proliferation, and detoxification of methylglyoxal (Thornalley, 1993). Glyoxalase enzymes are modified during phagocytosis, and the enzymatic reaction has been implicated as a virulence factor for neutralization of the immune response during invasion (Gillespie, 1978, 1981). It has been reported in sugar-limited environments, the fungus relies on fatty acid metabolism for growth (Sexton and Howlett, 2006). Accumulation of lipid in thick-walled resting cells at the expense of sugars is a key mechanism in yeast-to-mold conversion in black yeasts (Oujezdsky et al., 1973). Glyoxalase might thus play a central role in the response to varying conditions. In *Fonsecaea* the pathway is expanded in the clinical species of the bantiana-clade (Figure 7) suggesting that the glyoxal pathway cycle might be required for virulence during invasion, in addition to its role in surviving extreme environmental conditions (Teixeira et al., 2017). Caspases occur in the *Fonsecaea* core genome where they are synthesized as pro-proteins, possessing weak proteolytic activity (Madeo et al., 2002; Cheng et al., 2003; Abraham and Shaham, 2004) and also induce apoptosis enhancing pathogenesis (Douglas et al., 2015). Activation of apoptosis may lead to caspase-1 activation, providing a link between apoptosis and inflammation (Schumann et al., 1998; Lamkanfi et al., 2007). In the T class, a small GTPase family and an independent superfamily of GTP-binding proteins share enzymatic activity and play pivotal roles in cell division, protein synthesis and signaling (Paduch et al., 2001). Small GTPase—Ras type (IPR020849) is the most represented domain regulating cell growth, proliferation and differentiation (Table S1). The Ras family of GTPase proteins has been shown to control morphogenesis in many organisms, including pathogenic fungi such as *Cryptococcus neoformans* (RAS1) (Alspaugh et al., 2000), *Candida albicans* (CaRAS1 and CaRSR1) (Leberer et al., 2001) and *Aspergillus fumigatus* (rhbA) (Panepinto et al., 2003).

Heat shock proteins (Hsp) in the core genome (Table S1) are essential eukaryotic molecular chaperones, being first proteins that are up-regulated under conditions of elevated temperature (Lund, 2001). Especially, Hsp90 chaperones are unique in their ability to regulate a specific subset of cellular signaling proteins that have been implicated in disease, including intracellular protein kinases, steroid hormone receptors and growth factor receptors (Tamayo et al., 2013), which are likely mechanism of mammal infection where elevated temperature is an essential condition (Vicente et al., 2012).

The above discussed domains may explain the capacity to survive extreme conditions, of which living mammal tissue is one, but do not explain differences between species of the same clade. The significant predisposition observed in agents of chromoblastomycosis (Vicente et al., 2001, 2008, 2014) probably rests upon diversity enzymatic reactions. CAFE analysis of *Fonsecaea pedrosoi* and *Cladophialophora carrionii*, both causing this disease, evolve in opposite directions, as several domains expanded in the bantiana-clade appeared to be contracted in the carrionii-clade (Figure 7). This may demonstrate that members of different clades causing

chromoblastomycosis have evolved in different directions due to clade-specific ecological preferences, or perhaps more likely that the displayed domains *in toto* reflect phylogeny rather than ecology.

The CAZy Database is a powerful reporter of fungal lifestyles once the fungi degrade an enormous functional and structural diversity of complex plant polysaccharides. Zhao et al. (2013) revealed that most fungi that lack PL and tend to lose CE8, CE11, GH6, GH73, GH80, and GH82 families are saprobes; these were also observed in bantiana- and carrionii- clades. A wide variety of extracellular peptidases is produced to degrade a gamut of environmental substrate complexes, indicative for a less specialized nutritional status (da Silva et al., 2006; Sriranganadane et al., 2010). Species of the bantiana- and carrionii-clades produce enzymes involved in plant cell wall pectin and hemicellulose degradation. Besides, in both clades a significant similarity was observed among gene content related to carbohydrate metabolism and peptidase (Figure 6). It suggests that these fungi are able to degrade plant and animal substrates demonstrating a duality in lifestyle that could enable Chaetothyriales pathogenic species to transfer from environmental niches to animal material. The similarity of carbohydrate-active and protein degrading enzymes associated to the occurrence of additional virulence factors, which may support the tolerance to extreme environmental niches of the fungus (Teixeira et al., 2017), suggests an opportunistic tendency of *Fonsecaea* sibling species.

Primary fungal pathogens attempt to disrupt host cell homeostasis while avoiding and/or suppressing host recognition. In opportunists these mechanisms are not sophisticated and probably have emerged due to flexibility in nutrient acquisition (Dickman and Figueired, 2011) and extremotolerance (Moreno et al., 2017). Prenafeta-Boldú et al. (2006) and Casadevall (2007) suggested that this unfocused virulence explains the "dual use" determinants in unexpected agents of disease. It is likely that this principle also holds true for most black yeast-like fungi. However, as some common agents of chromoblastomycosis seem to have a significant predilection for this disease and are rarely found in the environment (Vicente et al., 2014) a certain degree of pathogenic adaptation cannot be excluded.

The subcutaneous infections by *Fonsecaea* and *Cladophialophora* species frequently result from a trauma from environmental sources. The muriform cell, considered to be a chromoblastomycosis-specific tissue form in humans, has been observed in cactus thorns infected with *C. yegresii* (De Hoog et al., 2007) and also *in vitro* in several environmental species (Badali et al., 2008). The *RSc1356* effector is involved in plant infection (Pensec et al., 2015) and its presence in *Fonsecaea* might support this hypothesis, although plant- and human-pathogenicity are almost mutually exclusive (De Hoog et al., 2000). The use of nitrogen and degradation of phenolic compounds that are also enriched in environmental *F. erecta* and *C. yegresii* (Table S7) are more likely causes of opportunism. The class of protein lectins (Table S3), which is implicated in many essential cellular and molecular recognition processes (Varrot et al., 2013) was present *F. erecta* isolated from plant material. De Hoog et al. (2004) stated that pathology on humans is coincidental, humans not being the primary hosts of these fungi.

The above hypothesis is partially supported by results of virulence testing using *G. mellonella* larvae as a model. Larvae infected by environmental *F. erecta* had a lower survival than those infected by clinical strains of *F. pedrosoi* (ATCC 46428 and CBS 271.37^{T}) and *F. monophora* (CBS 102248). In addition, *F. erecta* hyphae induced high levels of TNF-α (Figure 9), contributing to macrophage activation after phagocytosis. Macrophages activated by TNF-α increase their ability to control intracellular fungal growth, stimulate recruitment of inflammatory cells and stimulate the formation and maintenance of granulomata (Algood et al., 2005; Juhász et al., 2013; Gyurkovska and Ivanovska, 2016). Although *F. erecta* hyphae induced high levels of TNF-α, hyphal death was not observed, suggesting a higher level of extremotolerance. The higher virulence of strictly environmental *Fonsecaea* species does however not explain which the species which are commonly found on human hosts show lower virulence in the *Galleria* model.

Furthermore, based on PHI database the genes classified as lethal are mostly transcription factors (TFs) that orchestrate gene expression which determines life and functionality of the cell (Shelest, 2008) by controlling cellular signaling pathways and thus are key mediators of cellular function of fungi (Shelest, 2008; Wang et al., 2011).

The high number of domains related to enzymes and transporters reveals important mechanisms of nutrient acquisition and extremotolerance, constituting a genomic machinery that allows hydrolysis of recalcitrant components present in plant debris and suggests multiple survival strategies including mammal infection. Explanation of the observed differences in prevalence in the human host between closely related species appeared nevertheless impossible, as illustrated by the lower *Galleria* survival rate after infection with the non- pathogenic *F. erecta*. The large number of proteins (Figure 4, Table 4) with unknown function demands further investigation of these genes and their potential role in survival; small but crucial differences between these closely related fungi may have been concealed in the present set of proteins studied. Despite the close relationship in classical marker genes, *Fonsecaea* species are surprisingly different in their mitochondrial genomes, which in most fungi are highly conserved (Torriani et al., 2014; Jelen et al., 2016). Differences in routes of transmission allowing passage of properties acquired in the host to a next generation and thus, allowing evolutionary adaptation (Queiroz-Telles et al., 2017) are not easily revealed.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: VV, VW, LM, RR, ALB, SA, ES, SDH. Generation sequence data: HF, MZTS, VB, EB. Performed the experiments: GF, ALB, RdC, SA, SDS, MMFdN. Analyzed the data: VV, VW, AB, LM, FC, RR, AL. Contributed reagents/materials/analysis tools: RR, VV, FP, ES. Contributed to preparing the manuscript and revising it critically: VV, VW, AB, FC, AL, RG. Annotation and analysis of data; preparation, creation and/or presentation of the tables; graphics and figures: VW, AB, LM, FC, RR, AL, RG. Strains offered and/or Substantial contributions to the work FQ, ALB, SA, MMFN, SDH. Conceived and revised paper: JS, MT, MSF, MS, DA, MJN, VV, ES, SDH. Conception and design of the work and writing the manuscript. VV, VW, AB, ES, SDH.

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SUPPLEMENTARY MATERIAL

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CHAPTER III

Genomics and Virulence of *Fonsecaea pugnacius*, Agent of Disseminated Chromoblastomycosis

frontiers

Genomics and Virulence of *Fonsecaea pugnacius,* Agent of Disseminated Chromoblastomycosis

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Abstract

Among agents of chromoblastomycosis, *Fonsecaea pugnacius* presents a unique type of infection because of its secondary neurotropic dissemination from a chronic cutaneous case in an immunocompetent patient. Neurotropism occurs with remarkable frequency in the fungal family Herpotrichiellaceae, possibly associated with the ability of some species to metabolize aromatic hydrocarbons. In an attempt to understand this new disease pattern, were conducted genomic analysis of *Fonsecaea pugnacius* (CBS 139214) performed with *de novo* assembly, gene prediction, annotation and mitochondrial genome assembly, supplemented with animal infection models performed with *Tenebrio molitor* in *Mus musculus* lineages BALB/c and C57BL/6. The genome draft of 34.8 Mb was assembled with a total of 12,217 protein-coding genes. Several proteins, enzymes and metabolic pathways related to extremotolerance and virulence were recognized. The enzyme profiles of black fungi involved in chromoblastomycosis and brain infection were analyzed with the Carbohydrate-Active enZYmes (CAZY) and peptidases database (MEROPS). The capacity of the fungus to survive inside *Tenebrio molitor* animal model was confirmed by histopathological analysis and by presence of melanin and hyphae in host tissue. Although *F. pugnacius* was isolated from brain in a murine model following intraperitoneal infection, cytokine levels were not statistically significant, indicating a profile of an opportunistic agent. A dual ecological ability can be concluded from presence of metabolic pathways for nutrient scavenging and extremotolerance, combined with a capacity to infect human hosts.

1 Introduction

Melanized fungi known as black yeasts and related species belonging to the Herpotrichiellaceae family of the order Chaetothyriales are associated with different clinical pictures of diseases such as mycetoma, phaeohyphomycosis and chromoblastomycosis (Cañete-Gibas & Wiederhold, 2018). Chromoblastomycosis starts in the inoculation site of the etiological agent, leads to chronic acanthosis, and, triggered by the host's immune response, develops structures known as muriform cells (Vicente et al., 2017). While phaeohyphomycosis can be distinguished from other infectious syndromes by tissue invasion with pigmented hyphae (Thomas et al, 2018) and is often associated with necrosis.

Significant differences in pathogenicity and virulence between the main species *Cladophialophora bantiana*, *Exophiala dermatitidis*, *Fonsecaea pedrosoi* and *Rhinocladiella mackenziei* on the one hand, and with closely related environmental species have been reported. These species can grow at human body temperature or higher being able to cause systemic or disseminated disease, while many others, if causing infection, remain subcutaneous (Seyedmousavi et al, 2014; Teixeira et al., 2017). Cerebral infections by black

fungi are characterized by abscesses with hyphae in the tissue and therefore classified as phaeohyphomycosis. Such infections often lead to death of the patient despite combined treatment with antifungal drugs and surgery (Azevedo et al., 2015; Arcobello & Revankar, 2020).

Infection of humans by members of Herpotrichiellaceae is enabled by their stress tolerance and adaptability in their natural, environmental niche, the human host not being a preferential habitat (Gostinčar et al., 2018). The agents are saprobes in mostly still unclarified micro-habitats, and decompose organic matter for nutrition (Vicente et al., 2017). The high adaptability and invasive potential explain the relatively high frequency in animal hosts despite a low environmental occurrence. This potential for infection appears to be polyphyletic within the family Herpotrichiellaceae, as it differs between species (Vicente et al., 2017).

Fonsecaea sibling species differ significantly in their ecology and potential of infection (Vicente et al., 2014) which have been recognized as etiologic agents of chromoblastomycosis and phaeohyphomycosis in animal and human hosts, with species such as *Fonsecaea pedrosoi* and *F. nubica* associated with subcutaneous infection and ability to form muriform cell in human subcutaneous tissues , whereas *F. monophora* and *F. pugnacius* may also be involved in disseminated infection producing hyphal growth in the brain (de Azevedo et al., 2015, Vicente et al., 2012; Najafzadeh et al., 2009, 2011; de Hoog et al., 2004; Tanabe et al., 2004). Nine cases of primary brain infection by this species have been confirmed (Lucasse et al., 1954; Nobrega et al., 2003; Surash et al. 2005; Takei et al., 2007; Raparia et al., 2010; Koo et al., 2010; Doymaz et al., 2015; Varghese et al., 2015; Helbig et al., 2018). *Fonsecaea pugnacius* is exceptional by combining features of chromoblastomycosis and secondary neurotropic dissemination. The single strain known to date of the species presented muriform cells in subcutaneous tissue, but hyphae in the cerebrum (de Azevedo et al., 2015). This duality of local and invasive morphologies has not been observed in any other species associated with chromoblastomycosis or brain infection, suggesting that *Fonsecaea pugnacius* presents a unique pathogenic profile different from that of *Cladophialophora bantiana*, the main agent of human brain infection which presumably follows a pulmonary route (Ozgun et al., 2019).

In the present study, we sequenced the genome of the type strain of *F. pugnacius*, CBS 139214, and performed genomic analysis in order to identify the genomic relation between black fungi and neurotropism. In addition, we compared the enzymatous gene profile of *F. pugnacius* to other previously sequenced neurotropic species, including *C. bantiana*, *E. dermatitidis* and *R. mackenziei*. To obtain more insight into virulence, we evaluated animal

infection models by *F. pugnacius*, using strain CBS 139214 isolated from a human cutaneous lesion of a patient with disseminated neurotropic infection.

2 Methods

2.1 Genomic DNA extraction, sequencing and assembly

The *F. pugnacius* CBS 139214 strain (type strain) originated from a skin lesion (Azevedo et al., 2015) were obtained from the Westerdijk Fungal Biodiversity Institute collection, Utrecht, The Netherlands. The strain were grown in Sabouraud liquid media during 7 days at 28°C for DNA extraction according to Vicente et al., 2008 using cetyltrimethylammonium bromide (CTAB) and phenol-chloroform/isoamyl alcohol and the Microbial DNA UltraClean™ kit for purification. The the Nextera kit (IlluminaTM) and Ion Plus Fragment Library Kit (Thermo Fisher ScientificTM) were used to prepare the DNA libraries for sequencing based on the producer's guidelines. The FastQC (http://www.bioinformatics.babraham.ac.uk) was used for quality control analyses of sequence reads generated. The SPAdes assembler v3.10.0 (Bankevich et al., 2012) and FGAP (Piro et al., 2014) were applied for *De novo* assembly and gap closure respectively. The genome assembly was evaluated by BUSCO v4.0.2 using the 'chaetothyriales_odb10' dataset (Seppey et al., 2019) and the Bowtie2 program was used for assembly coverage mensure (Langmead & Salzberg, 2012).

2.2 Gene prediction, annotation and genomic analysis

The GeneMark-ES v4.39 (Besemer, 2001) was applied to predict the protein-coding genes using default parameter and RAFTS3 (Vialle et al., 2016) for automatic annotation with best hits comparison with self-score cutoff 0.5 using our internal database of sequences of *Fonsecaea* ssp. and related species from the Chaetothyriales order (Vicente et al. 2017; Moreno et al. 2018). The *F. pugnacius* functional characteristics were determined with GO enrichment analyses at a significance level of ≤ 0.05 according to Ashburner et al., 2000 using the InterProScan5 (Quevillon et al., 2005) to access the protein domain families. Phylogenomic trees based on orthologous clusters were obtained and generated using ORTHOFinder (Emms & Kelly, 2018). A phylogenomic tree was inferred for each and all-orthogroup trees were resolved by the OrthoFinder duplication-loss coalescent model (Emms & Kelly, 2018) and the final tree was constructed using the STAG method, present in the OrthoFinder pipeline (Emms & Kelly, 2018). The enzymatous gene profile of *F. pugnacius* and other melanized fungi causing brain infection (*C. bantiana, E. dermatitidis, F. monophora, Verruconis gallopava*, and *R. mackenziei*) were predicted with CAZY (Cantarel

et al., 2009) and MEROPS databases (Rawlings et al., 2016). In addition, numbers, classes and similarities were analyzed using an all-vs.-all similarity 40% ≥ search and e-value of 10^{-4} (Vicente et al., 2017).

2.3 Mitochondrial genome assembly and annotation

The SPAdes v3.6.2 program (Bankevich et al., 2012) was used for assembled and mapped the mitochondrial genome from the *F. pugnacius* sequencing reads previously aligned against the complete mtDNA of *Fonsecaea pedrosoi* CBS 271.37. The mitochondrial genome annotations were done based on Vicente et al. 2017 and Moreno et. al. 2018 using SILA (Vialle, 2013) and the final figure was produced by the software package Circos was used (Krzywinski et al., 2009).

2.4 Virulence assays in animal models

2.4.1 *Tenebrio molitor* infection

The *Tenebrio molitor* larval model was used to evalueted the virulence potential based on Fornari et al. (2018) using the parameters of survival and melanization after host fungal infection. The larvae was infected by the inocultion of 1×10^6 cells/mL in PBS solution above the legs and the ventral portion using as negative control the inoculation of sterile PBS solution and SHAM without physical damage (no treatment) using 10 larvae per group of inoculation, in triplicate. The larvae were kepted in darkness at 37 °C and mortality was monitored daily for 10 days and collected at 4 h, 24 h, 72 h, 168 h and 240 h post infection omitting the pulpae in the calculation (Scorzoni et al., 2013). Survival curves were plotted and statistical analyses were performed using the Log-rank (Mantel-Cox) test with Graph Pad Prism software and statistical differences were set at $p < 0.05$ according to Maekawa et al. (2015) and Vicente et al. (2017). The melanization was determined measuring the OD at 405 nm (Scorzoni et al., 2013; Perdoni et al., 2014). The fungal burden and histological analysis of caterpillars infected was performed according to Fornari et al 2018. The samples were homogenized in PBS solution with a TissueLyser (Qiagen, Hilden, Germany), inoculated on Mycosel agar at 30°C for 14 days and the number of colonies forming units (CFUs) of fungal per mL of solution estimated with some colonies re-isolated and sequenced to confirm the species ID. Moreover, the caterpillars samples were embedded in Adracanth gum solution (Fornari et al. 2018) immersed in liquid nitrogen and sectioned by steel blades in a cryostat (Leica CM 1850, Wetzlar, Germany) and stained with hematoxylin and eosin (HE) and observed on Axio Imager Z2 (Carl Zeiss, Jena, Germany) equipped with Metafer 4/VSlide automated capture software (Metasystems, Altlussheim, Germany).

2.4.2 Murine infection

The fungal burden and cytokine production evaluation was performed using immunocompetent mice as model, according previously described by Schneider et al. (2019), Rodrigues et al. (2015), Badali et al. (2011) and Bocca et al. (2006). The animals selected were old male Balb/c (6–8 week) and C57/BL6 mice, maintained under standard laboratory conditions with controlled temperature (23–25°C) with water and food at their disposal (ad libitum feeding), according to the Committee's recommendations of the Federal University of Paraná Ethics Committee (current approval certificate 1002).

The experiments were performed in triplicate using groups of six animals infected with F. pugnacius CBS 139214 and one negative control inoculated with sterile phosphate-buffered saline (PBS according to described by Fornari et al 2018 and Schneider in 2020. The animals were infected intraperitoneally or intradermally (per hind footpad) with 100 µL of 1 × 106 propagules or sterile PBS and were monitored weekly and sacrificed at 7, 14 and 21 days post-infection using CO2 anesthesia in an appropriate chamber (Fornari et al., 2018). Brain, lung, liver, kidney, spleen, footpad and blood were aseptically collected for analysis.

For fungal burden determination samples tissues were weighed, homogenized and diluted in PBS for culture as described by we based on Vicente et al. (2017). Results were expressed as number of CFU ± standard error of mean (SEM) per gram of fresh tissue, counting colonies from the seventh day until the fifteenth day. Cytokine production was measured from homogenized tissue obtained from infected and non-infected (healthy) animals by ELISA (Vicente et al., 2017). The cytokines interleukin-1β (IL-1β), TNF-α, interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1/Ccl2) were measured with kits purchased from eBioscience and used according the manufacturer's instructions. Results were expressed as pg of cytokine ± standard error of mean (SEM) per 100 milligrams of tissue. The infected tissue samples were fixed in 10% formalin, dehydrated in alcohol, and embedded in paraffin (Fornari et al. 2018). Serial 5-µm sections were stained with hematoxylin and eosin to visualize pathogen morphology.

3 Results and Discussion

3.1 *De novo* assembly

The genome sequencing of *F. pugnacius* CBS 139214 was performed using Illumina MiSeq and Ion proton producing 5,424,908 paired-end reads and 1,853,059 mate-paired reads, respectively. The final high-quality assembly comprised 386 contigs with 34,872,293 bp and 52% of G+C content. The genome size estimated is 34.8 Mb with average coverage of 48.75X and using 97% of the reads for draft assembling. The genome completeness, checked using BUSCO, revealed that the assembly had 98.8% completeness. A total of 6188

complete BUSCO genes were found, including 6176 being single-copy BUSCOs, of the 6265 BUSCO groups searched. 12 (0.2%) are duplicated BUSCOs genes, 39 (0.6%) are fragmented BUSCOs genes and 38 (0.6%) represent the missing BUSCOs genes. Sequencing data were submitted to GenBank (accession number WJFF00000000). In addition, 12,217 protein-coding genes and 35 tRNAs were predicted for *F. pugnacius* (Table 1). Expected values for *Fonsecaea* siblings were between 11,681 in *F. nubica* and 12,527 in *F. pedrosoi* (Vicente et al., 2017).

Table 1 - *Fonsecaea pugnacius* genome data assembly and related species of Herpotrichiellaceae.

Species	Strains	Genome Size (Mbp)	GC content (%)	Number of proteins	Genebank genome access
Fonsecaea pugnacius	CBS 139214	34.8	52	12,217	WJFF00000000
Fonsecaea pedrosoi	CBS 271.37	34.69	52.4	12,527	PRJNA233314
Fonsecaea monophora	CBS 269.37	35.23	52.2	11,984	LVKK00000000.1
Fonsecaea nubica	CBS 269.64	33.79	52.5	11,681	LVCJ00000000.1
Fonsecaea multimorphosa	CBS 102226	33.45	52.6	12,369	PRJNA233317
Fonsecaea multimorphosa	CBS 980.96	33.39	52.60	11,804	LVCI00000000.1
Fonsecaea erecta	CBS 125763	34.75	53.1	12,090	LVYI00000000.1
Cladophialophora immunda	CBS 834.96	43.03	52.8	14,033	JYBZ00000000.1
Cladophialophora bantiana	CBS 173.52	36.72	51.3	12,762	JYBT00000000.1
Cladophialophora carrioni	CBS 160.54	28.99	54.3	10,373	PRJNA185784
Cladophialophora yegresii	CBS 114405	27.90	54.0	10,118	AMGW00000000.1
Capronia epymices	CBS 606.96	28.89	53.4	10,469	GCA_000585565.1
Capronia coronata	CBS617.96	25.81	52.7	9,231	AMWN00000000.1
Exophiala dermatitidis	NIH/UT8656	26.38	51.47	9,578	GCA_000230625.1
Rhinocladiela mackenziei	CBS 650.93	32.47	50.4	11,382	JYBU00000000.1
Coniosporium apollinis	CBS 100218	28.65	52.1	9,308	AJKL00000000.1
Exophiala mesophila	CBS 40295	29.27	50.40	10,347	GCA_000836275.1
Exophiala aquamarina	CBS 119918	41.57	48.3	13,118	AMGV00000000.1

Comparing *F. pugnacius* genome size of 34.8 Mb with related species of Herpotrichiellaceae family (Table 1), some differences were observed: the *Cladophialophora immunda* genome is nearly 8.15 Mb larger, while the *Exophiala dermatitidis* genome is 8.43 Mb smaller, being the smallest genome in the family with 26.37 Mb (Teixeira et al., 2017). Genome sizes within the *Fonsecaea* genus were similar, i.e. between 33.39 and 35.23 Mb (Vicente et al., 2017). Judging from the phylogenomic tree (Figure 1), *F. pugnacius* clustered with the clinical representatives of *Fonsecaea*, in accordance with previous phylogenetic analyses of *Fonsecaea* and *Cladophialophora* based on internal transcribed spacer (ITS), partial beta-tubulin protein-coding gene (BT2) and cell division cycle 42 (CDC42) sequences performed by Azevedo et al. (2015).

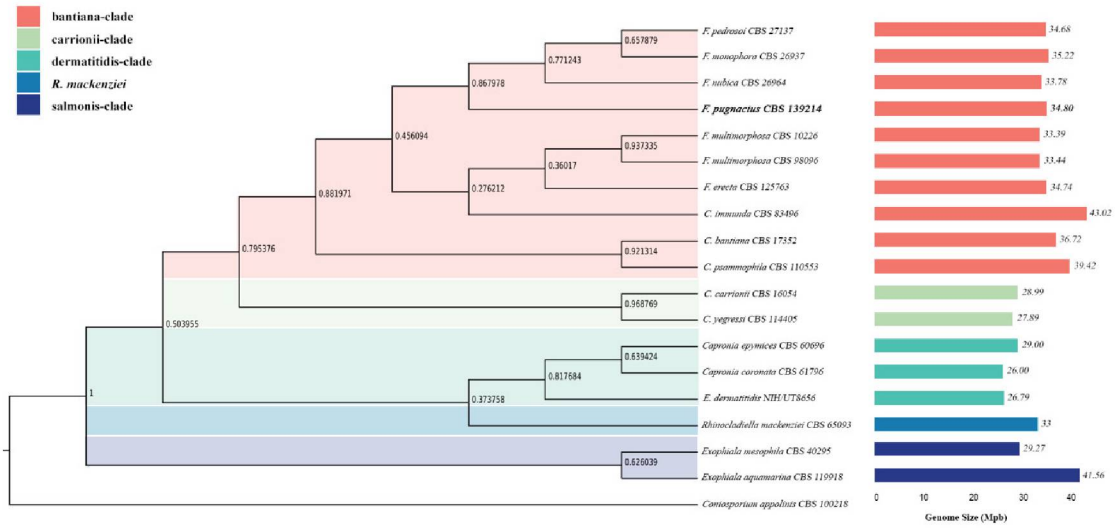

Figure 1: Phylogenomic tree based on the concatenated alignment of genomes of Herpotrichiellaceae family. Species names are given between phylogenomic tree and genome size bars. The genomes size is indicated and the colors are representing the clades of Chaetothyriales summarized in colored boxes.

The mitochondrial genome was assembled and 3 contigs were obtained with a total of 25,098 bp and a GC% of 25.49; the largest contig was 14,011 bp. There were 44 proteins in the mtDNA, i.e. 26 hypothetical proteins and 18 with known function (Figure 2). The proteins encoded by mitochondrial genomes of herpotrichiellaceous species were very similar, most being proteins involved in ATP synthesis and respiratory metabolism (Vicente et al., 2017).

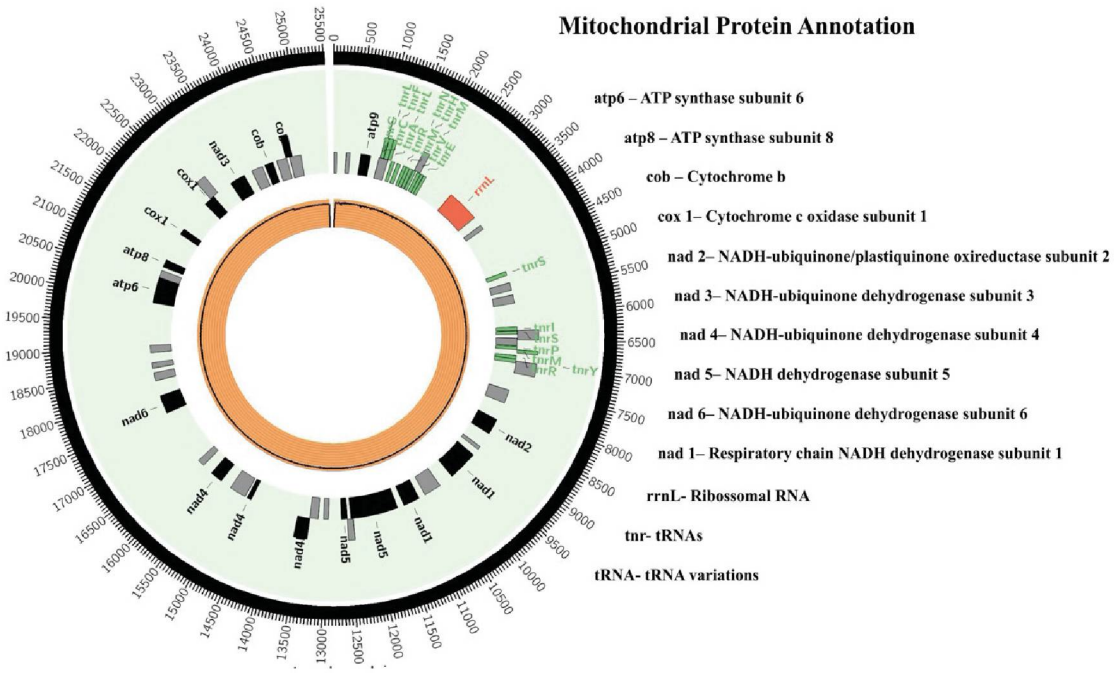

Figure 2: *Fonsecaea pugnacius* CBS 139214 mtDNA. Rectangles represent annotated genes: in red the rRNAs; in green the tRNAs; in black the other genes and orange inner circle shows reads coverage.

3.2 Protein-coding gene annotation and general characteristics

A total of 12,217 protein-coding genes were identified in *F. pugnacius*, of which 11,124 were annotated as hypothetical proteins and 1,093 proteins had inferred functions (Supplementary table 1). In the GO annotation, the annotated proteins were separated into 3 large groups: biological process, cellular components and molecular functions (Figure 3 and Supplementary table 1). Among proteins to which functions were attributed, various proteins were shared among the *Fonsecaea* siblings, such as proteins related to virulence in transporter families, proteins from the glyoxylate cycle, genes encoding proteins related to oxidative stress and involved in the detoxification of reactive oxygen species (ROS), cytochrome P450 monooxygenases (CYPs/P450s), heat shock proteins, proteins of melanin pathways, enzymes able to degrade aromatic carbon compounds, and others.

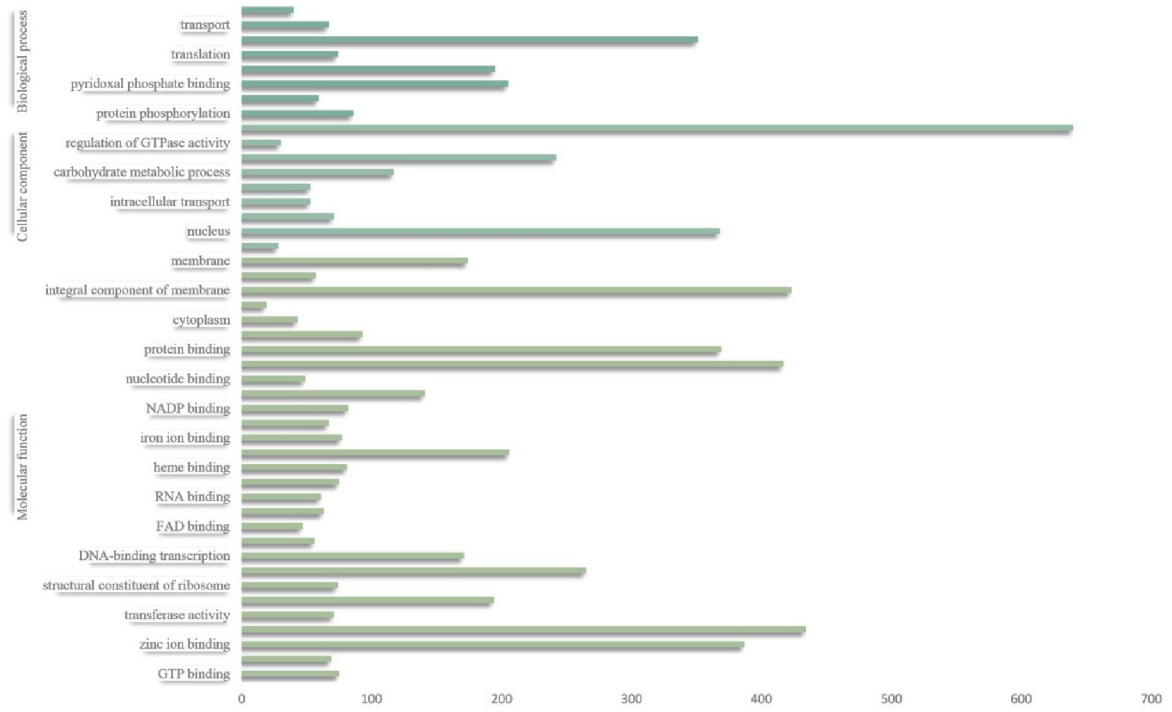

Figure 3: The most numerous gene families of *Fonsecaea pugnacius* based on Gene Ontology annotation separating proteins annotated into 3 large groups: biological process, cellular components and molecular functions.

Carrier families of zinc, iron, manganese and sugar have also been identified, i.e. MFS transporters and ABC transporters. In addition to basal metabolism, these carriers play a role in survival strategies. MFS is the largest family of transporters, ubiquitous to all living organisms and involved in the active excretion of antifungal drugs (Vela-Corcia et al., 2019). Reportedly, they also enhance antifungal resistance in *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* (Costa et al., 2014).

Sequences of proteins involved in the glyoxylate cycle were annotated: isocitrate lyase and malate synthase (Supplementary table 1). The glyoxylate pathway is a metabolic strategy for the synthesis of carbohydrates from carbon compounds, such as acetate and other degradation products from ethanol, fatty acids and poly-b-hydroxybutyrate (White et al., 2017). The glyoxylate cycle consists of a modification of the citric acid cycle (TCA), as it shares the same initial reactions of citrate and isocitrate generation but continues with the formation of succinate and glyoxylate (Dunn et al., 2009). This pathway has been associated with fungal virulence, since it allows energy production in environments where complex carbon compounds are poorly found (Lorenz & Fink, 2001) and has been described in infectious fungi such as *Rhinocladiella mackenziei* (Moreno et al., 2018), *Fonsecaea* siblings related to chromoblastomycosis (Vicente et al. 2017), *Beauveria bassiana* (Yang et al., 2016), *Talaromyces marneffei* (Thirach et al., 2007) and *Candida albicans* (Lorenz & Fink, 2001).

In *Paracoccidioides brasiliensis*, agent of another implantation mycosis, paracoccidiodomycosis, an increase of transcriptional levels of isocitrate lyase and malate synthase genes was reported in an infection model (Derengowski et al., 2008). This suggests a possible mechanism of adaptation of the fungus in response to the internal environment of the phagosome, which is poor in complex sources of carbon. The same function could be assigned to *Fonsecaea* siblings to explain fungal persistence inside macrophages, which, according to Queiroz-Telles et al. (2017), seems to be fungistatic (rather than fungicidal) against agents of chromoblastomycosis. In addition, it may be related to the ability of these fungi to survive on low carbon sources during brain infection.

Characterization of the partial genome of *F. pugnacius* revealed genes encoding proteins related to oxidative stress and involved in the detoxification of reactive oxygen species (ROS), such as alternative oxidase, manganese superoxide dismutase and cytoplasmic thioredoxins. Earlier reports observed that the alternative oxidase enzyme is present in the internal mitochondrial membrane of plants and some fungi and protozoa, in an alternative route of oxidation of the electron transport chain in cellular respiration (Duvenage et al., 2018). In the fungus *Neurospora crassa*, levels of the nuclear gene transcripts *AOX1*, which encode the alternative oxidase, increase when the cytochrome C oxidase pathway is inhibited. These results indicate activation of an alternative pathway, which the organism applies to correct conditions of oxidative stress and to decrease the production of ROS in respiration when competing with electrons of the classical oxidation pathway. Some studies have revealed that *AOX1* gene expression can be stimulated under stress conditions, such as low temperature and ROS low level, *AOX1* functioning as an antioxidant (Missall et al., 2004). Some pathogens resist to oxidative stress in the hostile environment of the phagosomes by the production of antioxidant enzymes that detoxify ROS, such as alternative oxidase, catalase and superoxide dismutase (Duvenage et al., 2018). ROS is an important cellular detrimental agent associated with the activation of immune response in human cells infected with fungi causing dermatomycoses or invasive mycoses (Castro et al., 2017).

According Vicente et al. in 2017, many CYPs/P450s enzymes revealed in herpotrichiellaceous are abundantly present in *Fonsecaea* siblings. Likewise, they were observed in *F. pugnacius*. Cytochrome P450 monooxygenases are heme-thiolate proteins with roles in oxidative functions, e.g degradation of xenobiotic compounds (Jawallapersand et al., 2014). Teixeira et al. (2017) noted that some black fungi are among the species in Ascomycota with the highest numbers of CYPs, with family expansion and diversification through gene duplication which could be explain the opportunism speciation. The authors suggest that these enzymes are may be involved in the metabolism of phenolic compounds and aromatic hydrocarbons, and of chemical compounds present in the human brain explain this species predilection.

Heat shock proteins are considered virulence factors because of their roles in thermotolerance and as molecular chaperones and are found in all prokaryotes and eukaryotes. They are classified based on approximate molecular weight (Tiwari et al., 2015). In *F. pugnacius,* the following families were identified: Hsp7, Hsp60, Hsp70, Hsp80 and Hsp90. Factors triggering the synthesis of heat shock proteins are oxidative or nutritional stress, UV radiation and exposure to chemical substances, indicating a protective role and aiding in cellular adaptation (Pockley, 2001).

Melanins confer resistance to heat, cold, enzymatic action and organic solvents, function as antioxidants and increase antifungal resistance (Nosanchuk & Casadevall, 2003). Main production route in *Fonsecaea* is the DHN pathway from acetate (acetyl-CoA) derived from glucose metabolism (Casadevall & Eisenman, 2012; Cunha et al., 2005). Melanin is a recognized virulence factor in several black and white pathogenic and opportunistic fungi, such as *Candida albicans, Cryptococcus neoformans*, *Aspergillus fumigatus*, *Exophiala dermatitidis*, *Paracoccidioides brasiliensis*, *Histoplasma capsulatum* and *Sporothrix schenckii* (Jacobson, 2000; Langfelder et al., 2003; Morris-Jones et al., 2003; Nosanchuk & Casadevall, 2003). Homologous proteins were identified in the herpotrichiellaceous black fungi *Exophiala dermatitidis* (Youngchim et al., 2004). In *F. pugnacius,* melanin-associated proteins related to DHN and DOPA pathways were observed (Suplementary table 1), as reported previously in *F. monophora* (Liu et al., 2019), such as tyrosinase, homogentisate dioxygenase and scytalone. Histopathological studies of organs such as brain, lung, liver and spleen in animal models did not clarify how the melanin production pathways are blocked in albino mutants of *E. dermatitidis* (Sudhadham et al., 2008) but the ability to block the oxidative burst effects increases significantly the pathogenic potential of *E. dermatitidis*, since the host is unable to eliminate it (Kumar et al., 2019).

Studies showed that fungi belonging to the genera *Fusarium*, *Penicillium*, *Aspergillus* and the family Herpotrichellaceae are capable of degrading aromatic compounds (Satow et al., 2005; Conceição et al., 2005; Teixeira, et al., 2017). Several species of black fungi have been isolated from hydrocarbon-contaminated environments (de Hoog et al., 2004). Fungi that are capable of assimilating monoaromatic hydrocarbons are enriched in the domestic environment (Sterflinger et al., 2001; Woertz et al., 2001; Prenafeta-Boldú et al., 2002). Prenafeta-Boldú et al. (2006) suggested physiological links between hydrocarbon assimilation by black fungi and certain patterns of brain infection. The brain contains small molecules that resemble alkylbenzene, phenylalanine metabolic products and lignin biodegradation intermediates, having structural similarity to neurotransmitters such as dopamine, which is catabolized in the brain (Fernstrom & Fernstrom, 2007). The tyrosine used for the biosynthesis of the neurotransmitters dopamine, noradrenaline and adrenaline has a phenylalanine as precursor and it is involved in the formation of melanin and

neuromelanin, dark pigments synthesized from L-dopamine for brain protection (Teixeira et al., 2017, Moreno et al 2018).

3.3 Virulence in animal models

The chromoblastomycosis agent *F. pugnacius* was described causing a secondary disseminated infection in an apparently immunocompetent patient. Among all agents of chromoblastomycosis, this is a unique type of infection, starting with a chronic skin disease and finally evolving to cerebritis. According previously reported by de Azevedo et al. (2015), during the implantation human infection, *F. pugnacius* was able to produce muriform cells in skin and hyphae in the brain. This dissemination apparent caused by conversion to another invasive morphology form have not been observed in *Fonsecaea* species. For example, in *F. monophora* which has also been associated with brain infection, but this occurs in a clinical event separate from the subcutaneous infection and therefore, the question is whether this infection ability is predictable only in *F. pugnacius* (de Azevedo et al., 2015).

Tenebrio molitor larvae were infected with inoculum concentrations of 5×10^6 cells/mL and observed while 10 days. The larvae infected with *F. pugnacius* exhibited higher mortality rates than control groups, PBS and SHAM (Figure 4A). *Fonsecaea pugnacius* presented a lower mortality rate than *F. monophora*, *F. erecta* and *F. pedrosoi,* as reported by Fornari et al. (2018). This indicates that *F. pugnacius* infection presents a slower development compared to *Fonsecaea* siblings involved in chromoblastomycosis, as well as to environmental saprobes. The fungal burden inside the larvae was assessed and presented significant numbers of CFUs, despite the low mortality rates caused by *F. pugnacius*: CFU values were initially countless and decreased along 72, 164 and 240 hours post-infection (Figure 4B).

Melanization of the larvae is an intracellular defense response and an effective barrier to infection. After 24 hours , the larvae infected with *F. pugnacius* presented a dark pigmentation, caused by the melanization in the hemolymph, which was observed during the entire 10-day period of analysis done by visual observation and spectrophotometry (Figure 4C).Similar results were observed by Fornari et al. (2018) in *Fonsecaea* siblings, showing maximum melanization with 24 hours post infection. The capacity of the fungus to survive inside the larvae was confirmed by histopathology; revealing melanized hyphae in tissue that had developed within 4 to 72 hours (Figure 4D).

Virulence tests using murine models Balb/c and C57/BL6 were conducted using two infection pathways: intradermal (per hind footpad) and intraperitoneal. In view of determination of fungal burden, *F. pugnacius* was recovered from kidney, lung and liver

after 7 days of incubation, indicating a certain preference of the fungus for these organs. After 14 days of intraperitoneal inoculation, *F. pugnacius* was recovered from the brain. At 21 days after infection, a muriform cell was observed in histopathology of the footpad (Figure 4E). The animal host infected intraperitoneally presented 1×10^2 and 1×10^4 CFU/mL in blood and organs (lung, kidney and spleen) after 7 and 14 days, respectively. The animals infected intradermally presented 2×10^6 CFU/g in the plantar cushion. The clinical aspects of these animals were evaluated, but no lesions, tissue necrosis or morphological alterations of internal organs were observed, except for plantar cushion swellings with (sub)cutaneous lesions (Figure 4F). Vicente et al. (2017) obtained similar results with Balb/c mice infected with *F. pedrosoi* by intradermal inoculation.

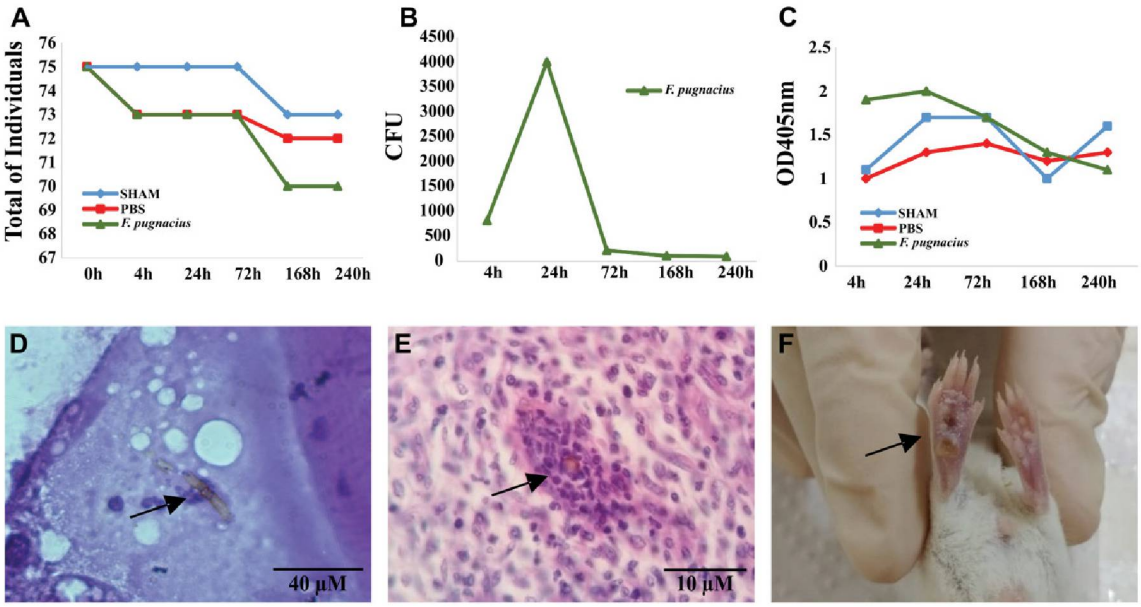

Figure 4: Virulence test of *Fonsecaea pugnacius* using animal models: *Tenebrio molitor* and Balb/c (*Mus musculus*). A) Mortality of the *Tenebrio molitor* larvaes at intervals of 4, 24, 72, 168 and 240 h post-infection. B) Fungal burden of *Tenebrio molitor* larvae tissues at intervals of 4, 24, 72, 168 and 240 h post-infection. C) Melanization of the hemolymph of *Tenebrio molitor* demonstrated by measuring the OD405 nm at 4, 24, 72, 168 and 240 h post-infection. D) Presence of melanized hyphae in *Tenebrio molitor* larvae tissue 72 h post-infection. E) Histopathology of footpad Balb/c tissue with presence of muriform cell after 21 days of intradermal infection. F) Necrotic lesion in footpad of Balb/c mice after 7 days of intradermal inoculation.

Immunological assays revealed insignificant levels of IL-2, INF-γ, TNF-α, IL-6 and IL-10, compared to what was described for other chromoblastomycosis agents including *F. pedrosoi* (Dong et al., 2018) and *F. monophora* (Jiang et al., 2018). The low immune response could be associated with a low virulent ability of *F. pugnacius*. Although the fungus

carries several genes with roles in pathogenicity and ability to survive in murine tissue, the immune system may not recognize their protein products, judging from absence of a cytokine response.

3.4 Potential virulence related to enzymatous genes

The observed brain infection might have been enhanced by the fungus ability to metabolize monoaromatic substrates as carbon source. Among these compounds, vanillic acid, phenyl acid, L-tyrosine, L-dopa, L-phenylanine, dopamine, sphingosine and others are present in mammalian brain (Prenafeta-Boldú et al., 2006). Subsequently we can verify in the *F. pugnacius* genome whether an enzymatic apparatus competent to degrade these compounds, i.e. with carboxylases, reductases, aldolases and kinases, is present. The species enzymatous gene profile was established with CAZymes and MEROPS databases. Carbohydrate-active enzyme analysis resulted in 476 genes encoding putative CAZymes, comprising 93 auxiliary activities (AA), 5 carbohydrate binding modules (CBM), 145 carbohydrate esterases (CE), 121 glycoside hydrolases (GH), 112 glycosyl transferases (GT) and zero polysaccharide lyases (PL). For MEROPS, 21 aspartic peptidases (A) were annotated, 59 cysteine peptidases (C), 14 trypsin peptidases (I), 133 metallo-peptidases (M), zero glutamic peptidases (G), zero asparagines peptidases (N), zero mixed peptidases (P), 189 serine peptidases (S) and 21 threonine peptidases (T) (Figure 5).

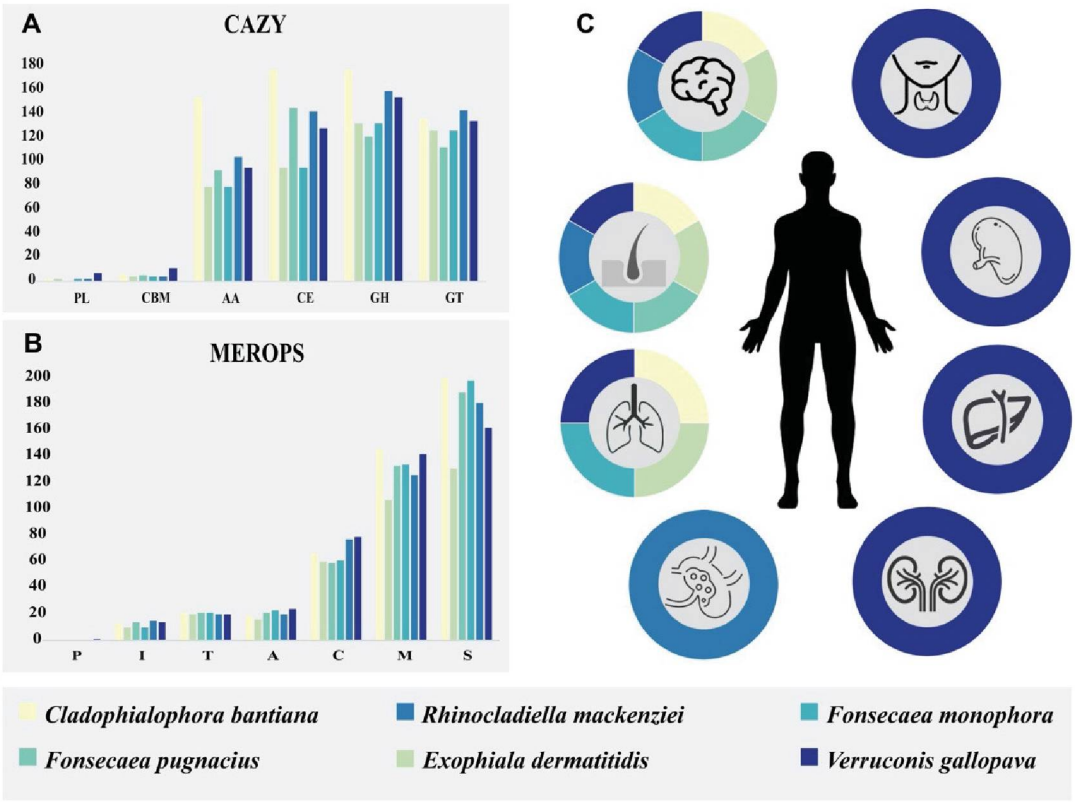

Figure 5: Species enzymatous gene profiles and epidemiology of human host pathogenicity are summarized in colored boxes. *Cladophialophora bantiana*: beige; *Exophiala dermatitidis*: light green; *Fonsecaea pugnacius*: medium green; *Fonsecaea monophora*: light blue; *Rhinocladiella mackenziei*: medium blue; *Verruconis gallopava*: dark blue. A) Carbohydrate-active enzyme (CAZY) annotation separated by classes. B) MEROPS annotation separated by classes. C) The species of black fungi related to subcutaneous and cerebral human infection and others affected organs: lungs, lymphatic system, kidneys, spleen, liver and thyroid gland.

We performed a comparative analysis of *F. pugnacius* with close related species, agents of (sub)cutaneous and brain infections: *Cladophialophora bantiana, Exophiala dermatitidis, Fonsecaea monophora, Rhinocladiella mackenziei* and *Verruconis gallopava* (Figure 5 and Supplementary table 2), that have been reported as agents of infection in others organs such as skin, lungs, lymphatic system, kidneys, spleen, liver and thyroid gland (Alabdely et al., 2020; Klasinc et al., 2019; Miossec et al., 2019; Wang et al., 2019; Grewal et al., 2018; Sideris & Ge, 2018 ; Mukhopadhyay et al., 2017; Jennings et al., 2017; Stokes et al., 2017; Azevedo et al., 2015; Geltner et al., 2015; Cardeau-Desangles et al., 2013).

The CAZy database is an exceptional reporter of fungal lifestyles degrading complex polysaccharides (Cantarel et al., 2009; Vicente et al., 2017). The GH class contains the majority of catalytic enzymes to degrade lignocelluloses (Cantarel et al., 2009) and seems to

be expanded in species associated with disseminated infection (Figue 5). Through this analysis (Supplementary table 3), we observed a high number of GH enzymes in all genomes analyzed, except for GH32 which was present in low numbers (*C. bantiana*: 3; *F. pugnacius*: 2; *E. dermatitidis*, *F. monophora* and *V. gallopava*: 1, *R. mackenziei*: 0). The CAZyme family GH32 are enzymes associated with energy storage and in extremotolerant species seems to provide energy for survival in extreme environments (Oren 2011). Additionally, the GH18 family, observed in these sibling species (strains), are chitinases with β-1,3-glucanase activities able to degrade chitin protein normally present in animal exoskeletons. Likewise, at minimal 29 GH families involved in the degradation of plant biomass (Li et al., 2011) were observed in the strains evaluated explaining the dual ability of these fungi to invade plant tissues.

Likewise, the high number Glycosyltransferases (GTs) can be related to neurotropism since these enzymes are responsible for the biosynthesis of glycoside (Cantarel et al., 2009). Some fungi are able to convert phenolic compounds into their glycoside correspondents (Tronina et al., 2013) and this ability may be derived from xenobiotic metabolism (Yang et al., 2014). The vertebrate brain contains a wide variety of gangliosides that are localized in specific cell types, such as on the surface of plasmatic membranes (Stanley, 2016). Gangliosides contain a fatty acid and a sphingosine base and are involved in neural functions such as memory formation, synaptic transmission, regeneration (Zitman et al., 2010). In cases of cerebral infection, the first symptoms described are locomotor difficulties and severe headaches (Koo et al., 2010; Azevedo et al., 2015). The GT41 family was the most numerous in our annotation, it is related to the metabolism of serine-threonine as indicated and involved in the process of subcutaneous infection (Vicente et al., 2017). Also, the GT2 family was abundant; proteins that act as chitin synthases (Brenton et al., 2006) are numerous in many fungi (Lee et al., 2018; Stone et al., 2018).

The AA CAZyme family is composed of lignolytic enzymes and are commonly found in plant pathogens. Similar to the GH and CMB families, they have a role in breaking down plant cell-wall polysaccharides (Lowe et al., 2015). The CBM family is composed of lectins and sugar transporters, while GH are glycosyltransferases such as lignocelluloses or chitinases (Yang et al., 2014). However, the GH class was more numerous in the species related to disseminated and cerebral infection (Figure 5). The lectins from yeasts and fungi have been associated with human infections involved in early stages of infection (Varrot et al., 2013), whereas in bacteria it seems to be involved in recognition of host glycan (Imbert &Varrot, 2008).

Polysaccharide lyases (PL) are enzymes able to cleave polysaccharide chains, this group presents many folds types (or classes), indicating that PLs are polyphyletic (Lombard

et al., 2010). Many fungi that do not have enzymes from the PL CE and GH families are saprobes, as these classes have enzymes related to cell wall degradation in plants (Zhao et al., 2013). This enzymatous gene profile suggested a dual ecological ability of these agents, in line with their extremetolerance and adaptability to variable environmental niches, which is a prerequisite for their opportunistic profiles.

Moreover, the peptidases play key roles in penetration of microorganisms into host tissue and are more involved in pathogen-host interactions (Ohm et al. 2012). Herpotrichiellaceous agents produce a variety of extracellular peptidases for the degradation of environmental substrates, indicating a nutrition not specialized (Sriranganadane et al., 2010; Vicente et al., 2017). The MEROPS S (serine) and M (metallo) peptidase families were the highest in number in the analyzed species causing brain infection. These two groups have been reported to be significantly enriched in transcriptome analyses of *E. dermatitidis* during infection (Poyntner et al., 2018). Among the Serine families, classes S33 and S9 were more numerous (Supplementary table 3), both involved in prolyl metabolism. Class S33 is a prolyl aminopeptidase family, which is not essential for growth but may confer a selective advantage allowing the organism to use proline-rich substrates (Iqbal et al., 2018). Teixeira et al. (2017) observed an expansion of the protein-degrading peptidase enzyme family M38 (isoaspartyl dipeptidases) in the bantiana-clade, the most numerous of metallo-peptidases family in the analyzed strains. This family is unusual in that the majority of characterized proteins are not peptidases but are associated with β-aspartic dipeptidase acting in the release of iso-aspartate residues from peptides (Palmeira et al., 2018).

Differences between closely related taxa are expected in ecology-related genes. The enzymatic repertoire of these fungi shows their ability to degrade a wide variety of substrates (Figure 5). This may suggest generalist and opportunistic ecology comparable to *Aspergillus* spp. permitting transfer from the environment to the animal host (Vicente et al., 2017), rather than pathogenicity where focused adaptation (Moran et al., 2011).

In addition, we analyzed genes codifying proteins related to degradation aromatic carbons pathway (Moreno et al., 2018; Teixeira et al., 2017) and the strains studied present a range of genes encoding homologous proteins (Table 2).

Table 2: Proteins related to degradation aromatic carbons pathway in *Fonsecaea pugnacius* and homologs in black fungi related to subcutaneous and brain infection.

	A	B	C	D	E	F
Fonsecaea pugnacius	gi|628298155		gi|915117215	gi|915113179 gi|915117218	gi|915082939	gi|915113183 gi|915117846 gi|915047106 gi|915117212 gi|915114200
Cladophialophora bantiana		XP_016622135.1	XP_016621839.1 XP_016624089.1 XP_016620669.1	XP_016620751 XP_016621838 1	XP_016619449.1	XP_016625963.1 XP_016624090.1 XP_016621840.1
Exophiala dermatitidis		HMPREF1120_09097	HMPREF1120_02976 HMPREF1120_03465 HMPREF1120_03826	HMPREF1120_03827	HMPREF1120_03438	HMPREF1120_03825
Fonsecaea monophora	AYO21_00136 AYO21_09571	AYO21_05363 AYO21_04106	AYO21_12165	AYO21_04852 AYO21_01974	AYO21_00578	AYO21_07675 AYO21_04851
Verruconis gallopava	PV09_08030		PV09_06763	PV09_04037 PV09_05381 PV09_02442		PV09_02443 PV09_02062
Rhinocladiella mackenziei		Z518_08486	Z518_05387	Z518_05995 Z518_05388 Z518_06895 Z518_09726	Z518_00072	Z518_02618 Z518_04953 Z518_04319 Z518_05993 Z518_05386 Z518_09725

Subtitle: A) Benzyl alcohol dehydrogenase B) β-carboxy-cis, cis-muconate lactonizing enzyme C) phenylacetate 2-hydroxylase D) homogentisate 1,2-dioxygenase E) maleylacetoacetate isomerase F) fumarylacetoacetase.

The presence of this homologous proteins explained partially the virulence of these strains by general factors like the presence of melanin in the cell wall, thermotolerance and the ability to assimilate of monoaromatic hydrocarbons (Moreno et al., 2018). The virulence of these strains is partially explained by general factors like the presence of melanin in the cell wall, thermotolerance and the ability to assimilate of monoaromatic hydrocarbons (Moreno et al., 2018). Furthemore, CYPs are involved in the degradation of aromatic hydrocarbons and xenobiotic metabolism, developing functions in the fungal pathogenicity and in the detoxification of exogenous compounds (Moreno et al., 2018).

The family Herpotrichiellaceae contains numerous black fungi that present tolerance to various types of stress, showing great adaptability to extreme environmental conditions,

presumably resulting from genomic information. Genomic studies of *F. pugnacius* showed the wide variety of genes involved in extreme tolerance and enzymes associated to occurrence of virulence factors. The survival capacity of fungi in animal models was confirmed by histopathological analysis and the presence of melanin in the host tissue. We have shown that *F. pugnacius* can colonize the brain and cause subcutaneous lesions with the formation of muriform cells in the murine model. An ecological capacity can be concluded from the presence of metabolic pathways for extremetolerance combined with the ability to infect human hosts. However, complementary molecular studies must be carried out in order to strengthen the connections between ecology and clinical profiles.

Gostinčar et al. (2018) found a link between (poly-)extremetolerance and opportunism, the ability to metabolize monoaromatic hydrocarbons improving human or animal disease. The order Chaetothyriales comprises opportunists, in which tolerance to various types of stress is associated with adaptability, presumably resulting in a large potential for habitat changes. The infection is as a side effect of the fungus adaptation to the human host, demonstrating that is not a favorable habitat, nor relevant to their evolutionary process. It defines opportunism against the pathogenicity, where the infection is advantageous for the fitness of the species. Most organisms considered opportunistic are unable to transmit from host to host, so specific adaptations will be lost with the cure of the infection, explaining the lack of complex virulence characteristics. Therefore, opportunistic infections can be considered an evolutionary end, which is unlikely to lead to true pathogenicity (Gostinčar et al., 2018).

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CHAPTER IV

Cytological and eletrophoretic karyotyping and de novo assembly of *Fonsecaea monophora*

In progress

Cytological and eletrophoretic karyotyping and de novo assembly of *Fonsecaea monophora*

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INTRODUCTION

Melanized fungi presents a different virulence profile in Herpotrichellaceae family, being related as chromoblastomycosis agent and primary brain agent of infection in humans (XIE et al., 2012). The *Fonsecaea monophora* was first reported in 1936, in chromoblastomycosis case in South America (MOORE & ALMEIDA, 1936).

F. monophora is the most variable clinical spectrum with a big versatility and ample adaptability, being a chromoblastomycosis agent with formation of muriform cells and primary brain infection agent presenting yeast cells. Although it is more frequent in immunosuppressed patients, it can affect patients with regular immunity (NAJAFZADEH et al., 2009).

However, the first reported case of chromoblastomycosis was initially attributed to C*ladosporium bantiana* (LUCCASE et al., 1954) and the second case, attributed to *Fonsecaea pedrosoi* (NOBREGA et al., 2003). Only in 2004, a new analysis allowed the identification of *F. monophora and* was described by Hoog, G.S; Vicente, V.A. & D. Attili (HOOG et al., 2004) showing that *Fonsecaea* siblings can only be differentiated by molecular methods (PINDYCKA-PIASZCZYŃSKA et al., 2014). Thus, after the description of *F. monophora*, the number of cases

attributed to this species increased significantly, it being the predominant agent in southern China (ZHANG et al., 2008).

Phaeohyphomycosis caused by *F. monophora* is a rare disease, only eight cases have been reported in the literature (LUCCASE et al., 1954; NOBREGA et al., 2003; SURASH et al. 2005; TAKEI et al. 2007; RAPARIA et al. 2010; KOO et al., 2010; DOYMAZ et al., 2015; VARGHESE et al., 2016; HELBIG et al.; 2018). The disease affects both sexes, occurring mainly in the larger age group with predisposing conditions and carries a high mortality rate (VICENTE et al., 2014). Patients that survived underwent long periods of treatment with various drugs such as voriconazole, amphotericin B, flucytosine and itraconazole (KOO et al., 2010).

In contrast to the recent advances of genomic field, the cytological information on chromosomes of *Fonsecaea* genus is unknown. The main purpose was standardizing a technique and determine the karyotype of *F. monophora* by cytological and electrophoretic karyotyping. In addition, to present the new genome assembly obtained with PacBio SMRT sequencing, aiming to complete the draft of *F. monophora* (BOMBASSARO et al., 2016).

MATERIAL AND METHODS

Fungal strain

The strain *F. monophora* was deposited in 1937 at the Westerdijk Center for Fungal Biodiversity, The Netherlands by M. Moore & F. P. Almeida. However, this species was described in 2004 by Hoog, G. S.; Vicente, V.A. & D. Attili. This isolate, denominated CBS 269.37 is the type strain and was used in all the methodologies described in this work.

Genomic DNA extraction

Methodologies in the literature and commercial kits were tested for obtain a high quantity and quality of DNA, the best performance was observed from the adaptation of the protocol of Vicente et al., 2008 described: the strain was grown in Sabouraud broth for 7 days in shaker at 280g. The DNA was extracted with MasterPure Yeast DNA Purification Kit (Lucigen) and for purification the Mobio Ultraclean Microbial DNA Kit (Qiagen). After, one more step was added, the DNA was treated with RNAse (400μg/mL) and Proteinase K (100μg/mL). The integrity of the genomic DNA molecules was checked using agarose gel electrophoresis overnight and Qubit DNA Assays (Thermo Fisher).

Genome Sequencing, Assembly and Annotation

The reads from SMRT sequencing are analyzed separated and together with the reads were assembled with SPAdes version 3.10.0 with Illumina and Ion reads (BOMBASSARO et al., 2016), following the steps described in the Figure 1.

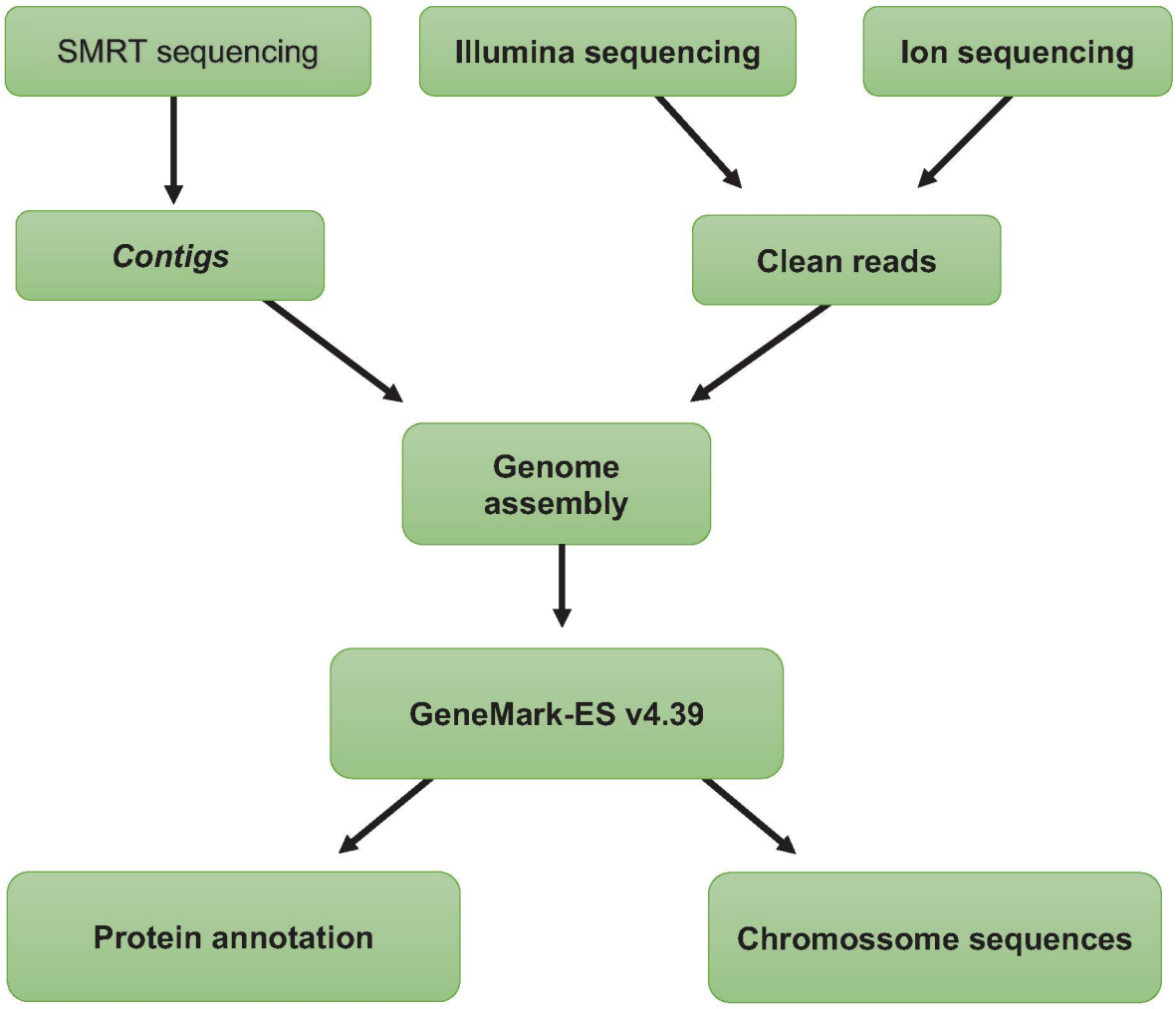

Figure 1. The workflow for the new genome assembly, annotation and chromosome identification.

The annotation was complemented and protein-coding genes were predicted with GeneMark-ES v4.39 (BESEMER, 2001). The chromosomes can be identified with the GeneMark annotation with the telomer sequence already described for many filamentous fungi (KEELY et al., 2005). The automatic annotation was performed by RAFTS3 (VIALLE et al., 2016) best hits comparison with self-score cutoff of 0.5 using a protein database available

(www.broadinstitute.org/annotation/genome/Black_Yeasts/). Protein domain families and functional annotation was accessed using InterProScan5 (QUEVILLON et al., 2005) and the tRNAs annotation used the ARAGORN software (LASLETT and CANBACK, 2004). Putative enzymes and peptidases coding genes using CAZY (CANTAREL et al., 2009) and peptidases coding genes using MEROPS database (RAWLINGS et al., 2015). The GeneMark annotation were used in order to identify the chromosomes.

Cytological observation

Mitotic chromosomes were prepared by modified protocols of the cytological karyotyping used for other fungi (TAGA et al., 2007). A conidial suspension of *F. monophora* in PBS solution (Phosphate buffered saline), in concentration $2x10^6$ conidia/mL, was incubated at 28°C for 18 hours. Three hours before the end of incubation, thiabendazole (10 mg/mL) was added to conidial suspension. After the incubation, slides were briefly dipped in distilled water to wash and immersed in fixative [methanol: glacial acetic acid = 9:1 (v/v)] for 30 minutes. For fluorescence staining of chromosomes, slides were mounted with a mounting solution containing 4,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) at 1 lg/mL each. Although DAPI alone can visualize chromosomes, DAPI/PI was additionally used expecting AT-rich regions to show up more clearly. Observations were made by UV-excitation using an epiflourescence microscope (Olympus BH2/BHS-RFC or Nikon Eclipse E600/ Y-FL) equipped with a 100 oil immersion objective lens. Fluorescent images were recorded using an Olympus digital camera (C5050-Z or DP-70) attached to the microscope with appropriate adapters.

Protoplast preparation

Protoplasts preparation was adapted according Eusebio-Cope et al. (2009). *Fonsecaea monophora* were grown in liquid-culture and used as like source of protoplasts. To obtain protoplasts, 150mL Sabouraud liquid was inoculated with conidia at 10^6 conidia/mL and incubated at 28°C for 7 days on shaker with 280g and were collected by filtration. After washing with $MgSO_4$ 0.6M, the conidia were suspended in enzyme solution containing 3mg Lysing Enzymes from *Trichoderma harzianum* (Sigma) and 10mg bovine serum albumin in 1 mL of $MgSO_4$ 1.2M, followed by incubation at 28°C for 2–3 hours. Protoplasts were twice washed in sorbitol 1M by centrifugation at 1200g for 3min, and finally suspended in STC (sorbitol 1M, $CaCl_2$ 100mM, Tris–HCl 100mM, pH 8.0). This suspension was immediately used or stored at -80°C.

Pulsed-field gel eletrophoresis

Protoplast–agarose plugs were prepared according to the method of Taga et al. (2007) with some modifications. Briefly, protoplasts stored in STC were washed twice with NaCl 1M by centrifugation at 1000g for 10min. Then, the pellet was suspended in SE (sorbitol 1M, EDTA 50mM, pH 8.0) at a concentration of $4x10^8$, mixed with an equal volum of low-melting-point agarose 1% (Sigma) dissolved in SE. The plugs were immersed in NDS (EDTA 0.5M, Tris–HCl 10mM, pH 8.0, 1% (w/v) sodium lauroyl sarcosinate) and Proteinase K solution (100 µ g/mL) at 37 °C for 36h, renewing the solution after 12 hours. After washed for three times of 30 min each in EDTA 50mM (pH 8.0), the plugs were stored in the same solution at 4 °C until they were used.

Pulsed-field gel electrophoresis (PFGE) was performed using a contour-clamped homogeneous electric field (CHEF) type apparatus (CHEF DR II, Bio-Rad). Chromosomes were separated in 0.8% agarose gel (pulsed-field certified or chromosome grade agarose; Bio-Rad). The running buffer was 0.5x Tris-borate-EDTA at 12°C. Chromosomal DNAs of *Aspergillus nidulans* ATCC 38163 was used as size markers. The electrophoresis conditions (pulse intervals and durations) were: A) 50 minutes, 45 minutes and 37 minutes, while 73 hours, 18 hours and 73 hours, respectively, with a voltage of 46V; B) 90 and 60 minutes while 72 hours each one. The voltage was 40V and the temperature was 12º C (Brody & Carbon, 1989). After the electrophoresis, the gel was stained with ethidium bromide (0.25 lg/mL) for 1 hour, followed by 1 hour washing in distilled water. Photographs were taken under UV illumination.

RESULTS AND DISCUSSION

DNA extraction and genome assembly

We perform several DNA extractions, using different methodologies based on CTAB, nitrogen and phenol methods (Table 1). But the best sample related to concentration and purification (highlight) was selected for the Pacbio SMRT sequencing. The standard method was the combination of the kits: MasterPure Yeast DNA Purification Kit (Lucigen) and Mobio Ultraclean Microbial DNA Kit (Qiagen) and the addition of one more step for DNA purification with treatment with RNAse (400 µg/mL) and Proteinase K (100 µ g/mL).

Table 1. DNA extractions of *Fonsecaea monophora* CBS 269.37 performed.

Year	Conc.(ng/$\mu\ell$)	260/280 ratio	260/230 ratio	Sample Vol. ($\mu\ell$)	Qubit Conc.Ng/$\mu\ell$
2015	649	2,04	1,14	75	
	634	2,04	1,39	100	
	752	2,11	1,84	100	
	862	2,09	1,53	75	
	684	2,05	1,45	75	
	669	2,05	1,46	75	
	338	1,71	0,76	75	
	1100	2,01	1,29	75	
	1382	2,09	1,56	75	
	624	1,98	1,24	75	
	1071	2,02	1,33	100	
	718	1,97	1,34	100	
2016	1997	2,12	2,23	150	
	1923	2,1	2,22	150	
	4592	2,13	2,25	150	93
	3745	2,15	2,25	150	
	1638	2,11	2,29	150	
	2385	2,09	2,26	150	
	1454	2,13	2,05	150	106
	1125	2,11	2,01	150	
	2511	2,11	2,04	100	
	2191	2,11	1,97	100	
	2972	2,04	1,91	100	
	1786	2,08	1,95	100	
	861	2,06	1,52	100	112
	782	2,06	1,58	100	
	1215	2,04	1,58	100	
	980	2,06	1,67	100	
	1068	2,12	1,89	100	
	719	2,05	1,55	100	
	7,1	0,72	0,06	100	
	6,8	0,64	0,06	100	
	5,1	0,48	0,05	100	
	93,2	1,9	1,42	150	13,02
	318	1,89	0,81	150	2,24
2017	1502,2	2,1	1,77	200	42,2
	465,6	1,85	0,83	200	15,03
	665,9	1,92	0,31	200	18,26
	291,1	1,69	1,89	200	14,89
	1533,4	2,12	1,83	100	88,8
	318,4	1,73	2	100	28,4
	729,4	2,01	0,33	100	82,6
2018	8919,1*	2,13	2,2	100	93
	3305,3	2,04	1,6	100	112
	6825,3	2,04	1,93	100	106

* Sample selected for the Pacbio SMRT sequencing

We sequenced the genome of *F. monophora* using PacBio technology to generate long reads. A total of 36,676 reads were obtained with *F. monophora* CBS 269.37 genome sequencing with PacBio SMRT sequencing. Early attempts to assemble the genome using Illumina had resulted in highly fragmented assemblies (BOMBASSARO et al., 2016). Together, PacBio, Ion Torrent and Illumina data add up to 9 million of reads. The result of this hibrid assembly was 2353 contigs with 52.34% of G + C content, 35191622 bp (35.1Mb) and N50 equal 57684pb. Although we obtained high quality samples, it was not enough to generate good reads from Pacbio SMRT sequencing and the new genome assembly showed no improvement. The melanin in the cell wall of *F. monophora* constituted a problem to be transposed in the DNA extraction, harms considerably obtaining a high quality DNA sample. Like other Chaetothyriales genomes have been sequenced using the Pacbio SMRT sequencing platform, such as *Exophiala lecanii-corni* (SCHULTZHAUS et al., 2019), Knufia petricola (SCHUMACHER et al., 2019) and others (not published yet); we will do new adaptations in DNA extraction protocol to perform a new genomic sequencing.

Fonsecaea monophora **karyotyping**

Cytological karyotyping is based on the microscopic observation of metaphase chromosomes, considering that is part of mitotic cycle and the chromosomes are present in high condensation state, being possible to individualization and consequent visualization. In the literature (TSUCHIYA & TAGA, 2001; EUSEBIO-COPE et al., 2009) is evident that the period of germination is determinant in the number of metaphases identified. For it, we evaluated the spore germination between 6 and 24 hours and the optimal spore germination time was determined in 18 hours of incubation of the spore solution at 28°C.

The thiabendazole was efficient on elevation of metaphase frequency. The total inhibition of spore development and an increase in the number of metaphases were observed after 3 hours of exposure of the fungus to the compound (Figure 2). Tsuchiya and Taga (2001) had previously observed the ineffectiveness of this compound in increasing the metaphase frequency for the fungi *Cochliobolus heterostrophus*, *Cochliobolus carbonum* and *Cochliobolus sativus*. On the opposite way, Tsuchiya et al. (2004) demonstrated the efficacy of this compound in disrupting the cell cycle in metaphase for *Neurospora crassa*.

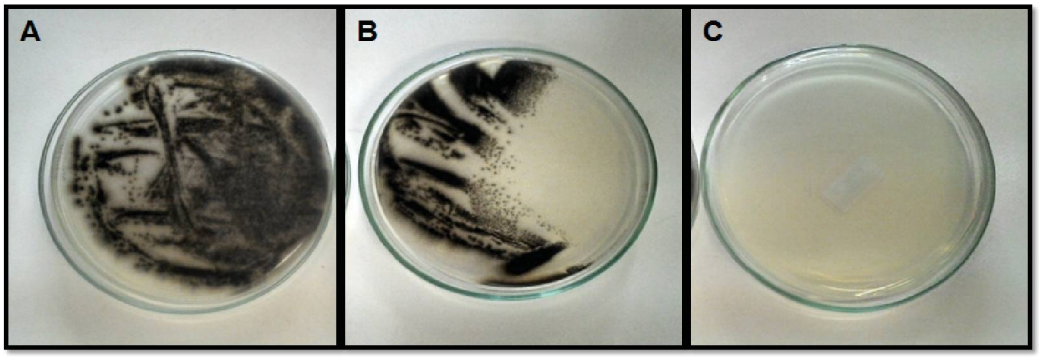

Figure 2. Culture plates with *F. monophora* exposed to thiabendazole. A) Plate with saline solution only 0.85% as like negative control. B) Plate with 2 hours exposure to thiabendazole and partial inhibition of fungus development. C) Plate with 3 hours exposure to thiabendazole with total inhibition of fungus development.

The chromosomes of *F. monophora* were stained with DAPI for fluorescence microscopy and observed. Based on the cytological observation, it was observed the *F. monophora* chromosomes (Figure 3A).

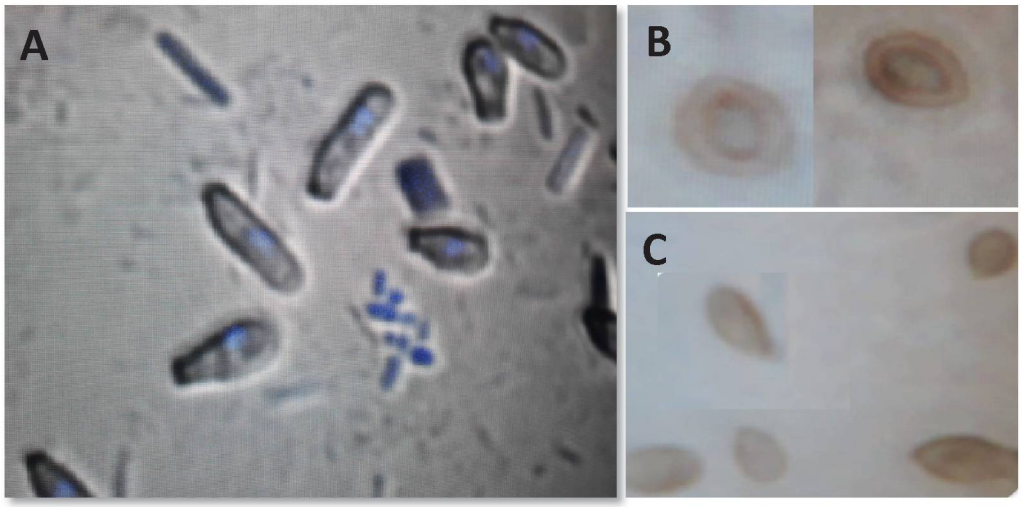

Figure 3. *Fonsecaea monophora* karyotyping. Cytological karyotyping: A) Conidia and chromosomes stained with DAPI. Eletrophoretic karyotyping (registered with optical microscope increased 1000x): B) Conidia pre-treatment. C) Protoplasts obtained after enzymatic treatment.

We have been trying to optimize the karyotyping methods for melanized fungi. This method step is still in progress. It was the unique image that was possible to infer the numbers of chromosomes in this species. According to this preliminary data we observed around 9 to 10 chromosomes in *F. mononophora*. The similar number

of chromosome (nine) was observed in the Ascomycota filamentous fungal *Fusarium solani* by Mahmoud and Taga (2012). Futhermore, thirteen different electrokaryotypes were identified in *Histoplasma capsulatum* (CANTEROS et al., 2015). Recently another clinical yeast, *Candida auris*, show chromosome numbers from 5 to 7 (BRAVO RUIZ et al. 2019). Sankaranarayanan et al., 2020 propose that AT-rich centromeres drive karyotype diversity in the *Malassezia* species complex through breakage and inactivation.

Here was the first time to present the chromosomes in *Fonsecaea* genus. Although it was visualized other techniques will combined in order to improve the data. In addition, the protoplasts were obtained by the enzymatic treatment. The cells were exposure to the enzymatic solution Lysing Enzymes Microscopic observation showed that protoplasts were individualized or aggregates (Figure 3 B and C). The protoplast formation depends on several factors, such as: type and concentration of enzyme solution, digestion time, osmotic stabilizer, pH and incubation temperature (MARCHI et al., 2006). The difference in the composition and structure of the cell wall of the fungi also is relevance in this process. The time of enzymatic reaction was also tested, demonstrating that after 5 hours, a decrease in the number of protoplasts was observed, probably due to degeneration of the first protoplasts formed (KIM et al., 2000).

Pulsed-field gel electrophoresis

The fragile conidias contained the lyse solution was homogenized with 2% low melting agarose (Sigma) and dispensed into molds for assembly of the agarose plugs in order to immobilize the spores inside of the agarose matrix. The complete disruption of the cell wall of the spores occur for action of the enzyme solution inside and outside of the plug since the high incubation temperature allows the interaction between the spore and the components of the solution.

The electrophoretic run showed that the chromosomes are present in the plug intact but is not possible the visualization of fragments of different size on agarose gel for *Fonsecaea monophora* and *Aspergillus nidulans* strains tested. We believe the chromosomes are not being fully released by the cell wall, because of this, the enzyme treatment time and the concentration of protoplasts is being increased. The time of action of lyse enzyme is crucial in order to form the protoplast cell and lyse of cell wall. According Mahmoud and Taga (2012), the PFGE method is important for size delimitation of chromosomes, that is not possible by cytology. However, other methods of karyotyping are being considered as southern blot or specific probes for telomeres, representing new perspectives for this study. In addition,

SMRT sequencing data will be complementary to this analysis, where chromosomes can be identified by searching for telomeres, helping to determine the number of chromosomes.

Since *F. monophora* is able to survive in human and animal host, and the environment, a functional analysis, genome research, as well as understand the biology of the agent are required to clarify the mechanisms involved during the fungal infection in chromoblastomycosis and phaeohyphomycosis. Mehabi, Gohari and Kema (2017) explain that chromosomal rearrangements have a potential evolutionary advantage for genetic diversity and adaptation to stressful environments. The characterization of chromosomes could connect the functional groups found in genome analysis and explain the distribution of predicted genes. In addition, allied to the PacBio SMRT sequencing can clarify and complete gaps found in genomic analyses. The karyotyping is still in progress but once this is established, it should will be useful not only for future genetic studies including linkage mapping and analysis of karyotype polymorphism.

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CHAPTER V

Final Considerations

Final Considerations

This work analyzed of two species of the genus *Fonsecaea* that have a closely pathogenicity profile: *F. monophora* and *F. pugnacius*, both are able to be an agent of primary brain infection and chromoblastomycosis in the same patient. This particularity inspired the development of this thesis based in genomic analysis and virulence tests.

The family Herpotrichiellaceae contains numerous melanized fungi present tolerance to variety of types of stress showing a large adaptability to extreme environmental, presumably resultant from the genomic information. The genomic studies of *F. monophora* and *F. pugnacius* showed the large variety of genes involved in extremetolerance and enzymes associated with the occurrence of virulence factors.

The capacity of the fungi to survive inside *Galeria melonella*, *Tenebrio molitor* and Balb/C was confirmed by histopathological analysis and by the presence of melanin in host tissue. We showed the *F. pugnacius* can colonize the brain and cause lesion in foot in murine model corroborating with the gene content of the metabolism for monoaromatic hydrocarbons degradation annotated in our genomic analyze. An ecological ability can be concluded from presence of metabolic pathways for extremetolerance combined with a capacity to infect human hosts.

The infection of human is a side effect of fungal stress tolerance and adaptability, the human is not the preferential habitat of this fungi. The opportunism is characterized when the infection is advantageous for the species suitability. But how the opportunists are generally incapable of the host-to-host transmission, any host-specific adaptations are lost, explaining the observed lack of specialized virulence traits.

Performing the fungal chromosomal characterization is a complicated work due to the nucleus size of the fungal cell and aggravated in melanized fungi by the presence of thick layer of melanin in the cell wall. Despite the difficulties in visualization, we obtained a first report of the stimated number of chromosomes of the genus *Fonsecaea* through cytological karyotyping. In the PFGE method, the enzyme treatment time and the concentration of protoplasts is being increased so that chromosomes can cross the cell wall and the size delimitation of chromosomes occurs. Several adaptations and other techniques are being evaluated so that we can elucidate the karyotype of *F. monophora*.

In addition, we believe that PacBio SMRT sequencing data can complementary to karytopyng data through the number of telomeres found in the assembled genome. These data together, will mean a great advance in the study of these agents, elucidating genome structure, demonstrating the chromosomal position of genes, signaling linkage groups, possible events of recombination, among other possibilities.

Although this study has provided a great advance in understanding the biology of these agents, a large number of proteins with unknown function demands investigation of these genes and their potential role in survival; differences between these fungi may have been ignored in the present set of protein. In addition, karyotype characterization will provide data on the chromosomal distribution of predicted genes and point to functional binding groups.

To finish, is important highlight, all the studies regarding about these diseases are essential to broaden our knowledge and to reduce the effects caused by these group of fungi to a neglected population.

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9. FLÁVIA DE FÁTIMA COSTA, MORGANA FERREIRA VOIDALESKI, GIOVANNA ZUZARTE CANDIDO, RAFAEL SOARES CORREA DE SOUZA, PAULO ARRUDA, DERLENE ATTILI ANGELIS, BRUNA JOCOMEL FAVORETO DE SOUZA LIMA, JADE MARIANE BARBOSA SOARES, LEANDRO FERREIRA MORENO, AMANDA BOMBASSARO, NICKOLAS MENEZES DA SILVA, RENATA RODRIGUES GOMES, FLÁVIO QUEIROZ-TELLES, ROBERTO TADEU RAITTZ, MAURO ANTONIO ALVES DE CASTRO, SYBREN DE HOOG, VANIA APARECIDA VICENTE. First report of isolation of *Cladophialophora bantiana* from an environmental source directed by metagenomic analysis.

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